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DEVELOPMENTS
in
INDUSTRIAL MICROBIOLOGY

A Publication of the Society for Industrial Microbiology

DEVELOPMENTS in INDUSTRIAL MICROBIOLOGY

Volume 2

Proceedings of the
Seventeenth General Meeting
of the
Society for Industrial Microbiology
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FOREWORD

The Society for Industrial Microbiology brings together people who share professional interests. A primary function of our organization is to help us communicate our interests with each other and with scientists in allied fields. It is not enough that some of us meet once a year to exchange ideas. These ideas must be made available for all. Hence, *Developments in Industrial Microbiology.*

This volume, the second in the series, is the proceedings of our Seventeenth General Meeting held August 28 through September 1, 1960, at Stillwater, Oklahoma, in conjunction with the annual meeting of the American Institute of Biological Sciences.

As with any union, ours is made more firm by the mutual responsibility for issuing a tangible product. This series of volumes is our baby. Each member of the Society can well take a proprietary interest in the results of our common effort. Also, each of us must see that the series not only continues, but continues to improve. We can best maintain the standards of these volumes by sustaining the quality of our annual meetings. It is our hope that the speaker-authors will not only give critical appraisals of what has been done in their fields, but also express opinions about where we go from here. These are the ideas we all look for, and from which we get the most stimulation.

This volume is possible only because the authors have entrusted their brain-children to our care. We have tried to preserve their style as much as possible, and hope nothing of importance has been lost in the editorial process.

It is my pleasure to acknowledge those who helped produce this volume. The prime movers, of course, were the committees, convenors, and speakers who participated in the Stillwater meetings. Brinton Miller, Secretary of the Society, was the expediter for this project, helping immeasurably in all phases. J. M. Sharpley collected the manuscripts and delivered them into the proper hands. The Plenum Press editors suggested a number of improvements and steered the volume through its manufacturing. The great bulk of the work fell on the Editorial Committee, who furthered the interests of the Society by giving unstintingly of their time and efforts. The Editorial Committee for this volume was Joseph Becker, Chester Koda, A. Kathrine Miller, and Leander S. Stuart. To them and to all who helped them, my heartfelt thanks.

Saul Rich
Editor and Chairman of
Editorial Committee for
SIM Proceedings,
1960-1961

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Editorial Committee for SIM Proceedings

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Saul Rich, Chairman	Connecticut Agricultural Experiment Station New Haven, Connecticut
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Chester Koda	Nuodex Corporation Elizabeth, New Jersey
A. Kathrine Miller	Merck Institute for Therapeutic Research Rahway, New Jersey
Leander S. Stuart	United States Department of Agriculture Washington, D. C.

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INTRODUCTION

James G. Horsfall

The Connecticut Agricultural Experiment Station, New Haven, Connecticut

The Society for Industrial Microbiology is young and, in its vigor, can afford to pioneer some new ideas in society management. Now we are pioneering some aspects of scientific communication, and I wish to say to the Society—*Congratulations.*

Symposia are not new, but the idea of devoting our major emphasis to symposia is, I think, new. The libraries are full of "original papers." Some are not so original, I grant you, but still we have a vast array of papers in our science and in the sciences ancillary to ours.

Up to now the highest status symbol in science has been the "original paper." This has been considered the essence of being scientific.

Perhaps, I may paraphrase St. Paul as he stood on Mars hill addressing the Athenians. "Ye men of Athens," said he, "In all things ye are too scientific." We in science have been so scientific as to fill the shelves with material "for the record."

It is high time that we "look at the record" and try to make sense of it. We now need to publish more papers that give coherence to our science.

A science is not an assemblage of facts, nor a library full of journals and reprints. It is integrated—a living entity; a melding of all the facts into an understandable whole; an evocative and provocative intellectual structure; a symphony if you will.

Most sciences, or, perhaps, I should say most segments of science are struggling with the problem of coherence. The stage of development to which most have attained is labeled as a "review" or "recent advances." "Reviews" are now being spewed over the scientific landscape.

Now, reviews and recent advances are good ventures. They aggregate, they collect, and to some degree, they collate the isolated fragments of data in the proliferated original papers.

I submit that reviews are even now obsolete. They are not sophisticated enough. A poker hand is what you call it. When one is asked for a review, he is asked, by and large, for a routine job. He is asked to dig up all the literature and pass it in review. We get a review and nothing more.

We need something more. What we need is syntheses. What we need are new designs constructed from the bricks at hand. The time is ripe for the rise of architects in science. We need those who can study the information available and tell us what it means. Van't Hoff synthesized the structure we know as osmosis from data already "in the record." Those who put it there did not recognize what they had. Van't Hoff did. He was a synthesizer.

In SIM, we, too, have struggled with the same problem—and we are doing something about it. We are devoting the major share of our annual meeting to critical discussions of our main areas of interest. We refer to our discussions as symposia, and we print our proceedings in hard-backed books of which this is Volume 2.

This is real progress. In this we are pioneers, and in this we can take honest pride.

I hope that we can go still further. Symposium is a good term, meaning to talk together. But we want more than that. While we talk together we want to wind up, I am sure, with a synthesis of the field. Let us ask for syntheses.

Let us urge each author to sort and resort, shuffle and reshuffle the data available to him until he can design a beautiful new structure from all the bricks and mortar. I would ask him to face up to controversies, tell us what he thinks, straighten us out to the best of his ability.

This series is a beautiful pioneering venture. Let us venture still further into the frontier of scientific communication.

PETROLEUM MICROBIOLOGY

J. Miles Sharpley, Convenor

Panelists: Ray C. Allred
Gordon P. Lindblom
R. L. Raymond
Don O. Hitzman

INTRODUCTION

J. M. Sharpley

Buckman Laboratories, Inc., Memphis, Tennessee

It was originally intended for the convenor merely to introduce the speakers for the Symposium on Petroleum Microbiology. It has become apparent, however, that a great deal of interest has been aroused concerning some of the activities in petroleum microbiology. Since it is not feasible for this symposium to do more than touch briefly on some areas of current interest, it was considered that a brief outline of the general activities in the field might serve a useful purpose.

Petroleum microbiology is far from a new field. There has been scattered work reported on the bacterial attack of hydrocarbons since the time of Beijerinck. Sohngen published the first paper on bacterial utilization of methane in 1906. Until recently, it would be fair to describe petroleum microbiology as an academic activity. Traditionally, it has been a rewarding field of work for investigators interested in oxidative metabolism. Much current industrial petroleum microbiology is founded on scattered and sometimes obscure research papers discussing the activity of a given species of microorganism on one of the shorter chain hydrocarbons.

Today, the field of petroleum microbiology is rather sharply divided between academic and industrial microbiologists. There is some fine work in progress in academic institutions. Our immediate interest, however, lies with the efforts of microbiologists allied with the petroleum industry.

There are possibly ten or twelve professional microbiologists currently employed by oil companies whose efforts are largely in petroleum microbiology. There are about the same number of professional petroleum microbiologists employed by chemical manufacturing companies. In addition to these microbiologists, there are possibly fifty people of various disciplines and degrees of training who from time to time work in petroleum microbiology. Petroleum microbiologists form a very small group but characteristically are active in research and publication.

In general, the activities of petroleum microbiologists may be roughly classified as follows:

Oxidation of hydrocarbons
Deterioration of drilling muds
Prevention of microbial growth in secondary recovery systems
Exploration
Corrosion
Cutting Oils
Manufacturing

The oxidation of hydrocarbons is characteristically involved in any aspect of petroleum microbiology. Hydrocarbon oxidation is separated in the list above to emphasize the industrial interest in microbial utilization of mixed hydrocarbons, such as JP-4 fuel, and the potential industrial utilization of certain microbial hydrocarbon oxidations. Some of these aspects of hydrocarbon oxidation will be discussed during our symposium.

The deterioration of drilling muds will not be discussed. Thus, a definition and a brief description seem in order. Drilling muds are complex mixtures of clays containing additives that may be attacked by microorganisms. Drilling fluids are used for cooling and lubrication of the drill bit, removal of cuttings, and prevention of well caving. Microbiological work on drilling muds often is not true petroleum microbiology. However, it is usually considered a facet of petroleum microbiology because of the intimate association with the industry.

The microbiology of secondary recovery systems will be discussed in detail by one of the symposium members. The secondary recovery of oil is accomplished by forcing water under high pressure into the oil-bearing reservoir. The growth of bacteria in secondary recovery water causes operational difficulties and ultimate decrease in oil production. A good deal of effort is currently devoted to this phase of petroleum microbiology.

Exploration techniques using microorganisms will be discussed in some detail by one of our panel members. Microbiological exploration may be defined as a technique for detecting hydrocarbon gases seeping through earth structure from a petroleum reservoir.

Microbiological corrosion is an important aspect of petroleum microbiology. Some microorganisms, such as *Desulfovibrio desulfuricans,* can establish electrochemical corrosion cells. A microorganism producing a hydrogenase will utilize hydrogen at the cathode of a corrosion cell. When hydrogen is utilized, current will flow, and electrochemical corrosion will occur.

The microbiology of cutting oil is probably the best generally known facet of petroleum microbiology. At this time there appears to be little research being done in this field. The need for preservation of cutting-oil emulsions is well established.

Bacteria appear to predominate in most of the microbiological problems associated with the petroleum industry. Fungi are common in certain specialized environments and are usually very active in attacking hydrocarbons. The microflora in secondary recovery systems, fuel oils, cutting oils, exploration, and corrosion have been very poorly characterized. It is often found that the majority of microorganisms isolated from these industrial situations are unidentifiable using available taxonomic schemes. It is very common to isolate and identify a microorganism from a petroleum environment that will not grow, or with which the original microbiological problem cannot be reproduced. The characterization of microflora in all fields of petroleum microbiology is a major unexplored area of investigation.

The effect of pressure on the metabolism of bacteria and fungi occurring in oil reservoirs is essentially an unexplored area. There has been some work reported but this is distinctly another major unexplored area of investigation.

As is true in other industrial environments, the metabolism of mixed cultures is unknown. There appears to be little doubt that mixed genera of bacteria or fungi often have different reactions and metabolic rates than the single species in the same environment.

Isolation of cultures and culture techniques in general are imperfect in petroleum microbiology. There seems little doubt that environmental changes in many of the situations alter the cultural requirements of microorganisms. For example, the oxygen requirements of microorganisms that have been accepted for many years as aerobic now seem in doubt. Microorganisms such as *Sphaerotilus*, *Gallionella*, and methane-oxidizing bacteria are considered by bacteriologists as being strictly aerobic. Yet all of these microorganisms grow prolifically in water that contains no detectable oxygen. This would indicate that these microorganisms are growing in oxygen concentrations of less than 0.1–0.3 ppm. A better field technique for detecting dissolved oxygen is needed and basic research will be required to clarify the oxygen requirements of microorganisms.

Much of the work concerning the occurrence of bacteria in oilfield environments has unfortunately been based on shipped water samples. It has been adequately shown by many investigators that microbiological changes occurring in shipped or stored water are of great magnitude. The occurrence of pseudomonads in shipped water samples, for example, is greater than the occurrence of pseudomonads in natural waters examined immediately after sampling. There is a major need for techniques allowing the shipping and transportation of samples without changing the microbiological flora.

The problem involved in controlling microbial damage by use of bactericides and fungicides in the petroleum industry is often a difficult one. The mechanisms of action of bactericides and fungicides in the presence of oil and water containing very large amounts of dissolved solids are apparently much different from the usual mechanisms. It is a common observation that many of the reliable fungicides and bactericides are essentially inactive when used in these specialized environments.

The problems in industrial petroleum microbiology are numerous and immediately absorb the interest of those who become involved in them. We hope that this very rapid and very brief survey of some of the problems encountered will aid in orientation during the remainder of this symposium.

MICROBIOLOGICAL PROBLEMS IN SECONDARY OIL RECOVERY

(ABSTRACT)

Ray C. Allred

Continental Oil Company, Ponca City, Oklahoma

In secondary oil recovery by waterflooding, water is injected into a partially depleted oil-bearing formation to force additional oil from that formation. If the injection water contains bacteria, insoluble products of bacterial metabolism, or suspended solids, the sand face of the injection well can become plugged. The result could be a decrease in water injection rates with a subsequent loss of oil production.

There are many types of microorganisms capable of causing plugging problems in secondary recovery waterflooding systems. They include iron bacteria, slime-forming bacteria, sulfate-reducing bacteria, algae, and in some cases sulfur bacteria. Plugging problems can result from the presence of large numbers of microorganisms or insoluble products of microbial metabolism.

Microbial control can be established only after a critical survey of the problem waterflood. The fact that a chemical has been used successfully on one waterflood does not mean that it will be effective on another. There is no universal bactericide. Microbicides that have been used effectively include amines, quaternaries, chlorinated phenols, imidazolines, and organic sulfur compounds.

MICROBIOLOGICAL ASPECTS OF ORGANIC GEOCHEMISTRY

Gordon P. Lindblom and Marcia D. Lupton

Geological Division, Jersey Production Research Company, Tulsa, Oklahoma

INTRODUCTION

Most of the research in microbiology is concerned with trying to find answers to the question, "What do bacteria do?" In recent years a new breed of researcher, the "microbial ecologist," has emerged. By striving to work out and understand the complex relationships among the chemical and biological parameters within which microbes live, he seeks to answer the more specific question, "What do bacteria do, chemically, in nature?"

From our very first contacts with bacteriology, we are all aware of such fundamental concepts as the cycles of carbon, nitrogen, sulfur, phosphorus, and other elements. Further, that microbes are associated with the breakdown or alteration of natural organic material is accepted by all. The destruction or alteration of organic molecules in soils and sediments is perhaps the major contribution of microorganisms to the chemistry of the earth, but the processes and mechanisms here involved are some of the best-kept secrets of nature.

Problems relating to plant growth and fertility of the soil have been studied by chemists and microbiologists for many years. Yet there is only now emerging any understanding of the details of the individual chemical reactions that are involved, and little is known about the contributions of specific organisms to changes in soil organic matter. There are two reasons for this. One is the recognized difficulty of working with mixed cultures, and the other is the problem of seasonal changes in microbial populations, which make it difficult to gain any understanding of continuous soil processes in the laboratory.

Processes occurring in both fresh-water and marine sediments are even less understood. Though physicochemical conditions in sediments are quite different from those in soils, they are also more static. Even so, biological study of these muds has lagged, perhaps because of a complex chemistry, because of the remoteness of most good sampling areas, but primarily because of the scarcity of workers interested in this field.

In the study of the microbe's contribution to organic geochemistry, the problem is essentially this: What are the molecular changes brought about in sediments by microorganisms living therein, by what mechanisms do these occur, and how do various environmental factors bear on these processes?

To try to understand what is going on, we must consider three separate factors—the microbial, the biochemical, and the ecological. New experimental and analytical techniques are required to unravel the complexity of the interactions of these factors in varied environments.

Recent marine and other aquatic sediments are of particular interest for such a study because there is a tremendous organic productivity in lakes and oceans, which provides a large reservoir of organic detritus in their sediments. Partially degraded terrestrial organic matter contributed by the entrance of rivers provides additional substrate material for the organisms in these sediments. In addition, sediments are much less influenced by external forces than are soils.

Our purpose here is to present a working philosophy of research on the microbiology and biochemistry of recent marine sediments, and to stimulate the interest of others in the research needs in this field.

We will discuss a general approach to the problem as some of us see it, then describe a possible experimental procedure, and finally present some preliminary results which will illustrate the type of information we are obtaining.

THE PROBLEM

Fundamental Hypotheses

Our approach to the study of sediment microflora is based on several closely related working hypotheses:

1. The depositional environment is an important variable in effecting the distribution of both microorganisms and organic matter.

By this we mean that the physiological or biochemical types of organisms found in a given kind of sediment (carbonate, deltaic mud, coastal bay, sandy, evaporitic, etc.) are more or less specific for that environment. The physical conditions will determine the various forms of plant life that will be present, and thus also the type and concentration of the molecules which eventually become the food for organisms.

2. The physiology and metabolic potential of the groups of heterotrophic microorganisms differ from one depositional environment to another.

It is obvious that the organisms in a given environment must be able to adapt to the available substrates to survive. Since the substrates available differ in amount and distribution, a group of organisms from one environment will be found to possess certain patterns of enzyme activity which organisms from another environment do not possess.

3. The type of chemical activity of a group of microorganisms is determined by the type of organic material available.

The reactions carried out by organisms in a given sediment must be dependent on the raw material which they have to begin with.

4. The microorganisms in a given ecological niche undoubtedly interact with each other metabolically, perhaps being adapted in deeper sediments to a degree of symbiosis.

If various types of organisms are to survive together in deep sediments, either there must be no competition for food material or the members of a given microbial community must develop methods of co-existence. For example, an organism may perform only certain reactions when it is in association with other organisms in an environment of minimal nutrition. If some arrangement such as this did not exist, one could not find multiple types of organisms in sediments thousands of years old.

Microbiology

The organisms with which organic matter in mud is associated must have some effect on it. With respect to a single molecule, the organisms might destroy it, i.e., metabolize it to cell material, carbon dioxide, water, etc.; utilize it partially, leaving a remnant behind; or ignore it entirely in their metabolism. Therefore, knowledge of the distribution of bacterial physiological types at different levels in sediments is important to the problem.

Biochemistry

Knowledge of what specific organic molecules are present in a natural environment is necessary to draw legitimate conclusions about the role of the organism from artificial laboratory tests. With this knowledge it may be possible to determine in great detail what occurs in nature. Indeed, it should not be too much to hope that such information may eventually result in some sort of controlled artificial reconstruction of the environment, from which a wealth of additional information might be obtained. Therefore, we feel it is essential to study the distribution of molecules as well as of microbes.

Ecology

We must continually be aware that the organisms we study exist in nature in association with other microbes. The common laboratory method of the "pure culture" is criticized as being not at all like the natural condition, since the reactions taking place there may, in nature, be modified or unimportant, or indeed may not even occur. However, well-controlled investigation of the properties of pure cultures will provide excellent background on which mixed-culture experiments can later be built.

This investigation may involve the determination of the nutritional requirements and metabolic potential of individual organisms from a given microbial community. For example, determination of the amino acid requirements of a group of bacteria is an effective beginning to an understanding of the fate of the various amino acid molecules in their vicinity.

Another important point to keep in mind is that standard laboratory practice is to "overfeed" bacterial cultures. Biochemical data will also provide information on the true nutrient levels in the vicinity of a given microbial community; and in sediments this level is often found to be astonishingly low. Metabolic patterns are modified by the association of organisms, and organisms other than bacteria, fungi for example, may contribute significantly to the biochemical ecology of the community. The subject of nutrition in the study of microbial ecology has been discussed recently in more detail by Gibson (1957).

EXPERIMENTAL PLAN

The actual research effort requires sampling from a variety of environments, measurement of physical conditions, and determination of microbial populations in the field. It cannot be stressed too strongly that "getting one's feet wet" is an essential part of this whole business. Nobody, least of all a microbiologist, can study ecology unless he has an adequate picture of the environment with which he is working, and an appreciation for the natural conditions he is trying to interpret.

The locations for study must be selected carefully and the environments sampled must be geologically well defined in order that the data can be analyzed from an environmental point of view. Proper precautions against contamination are necessary when samples are being taken, and data on populations must be obtained immediately in order to be valid, as has been shown by several workers. Although the methods used in the field must be adequate, they must also be simple and fairly rapid. However, since this paper is not concerned with techniques, they will not be discussed here.

In many cases, extraction of the major biochemical fractions (proteins, carbohydrates, lipids) should also be done in the field, with definitive analyses of the extracts for individual molecules being left until later.

Following the field work, the organisms can be isolated from the enrichments in the laboratory. As each organism is isolated, it can be preserved by freeze drying, and its metabolic potential can be determined later in a series of tests. Patterns of biochemical ability appear when groups of organisms from a given sampling area are compared with those from another area. It is important here to differentiate between what an organism *will* do in an artificial test in laboratory media and what it *might* do in nature. Most microbiologists are aware of this but assumptions that equate laboratory with nature are unfortunately still common.

Mixed-culture studies of microbial communities are the next obvious step, using information obtained from biochemical extractions as a basis for determining nutrient levels. At this stage, use of continuous-culture systems, with frequent analyses for reactants and products, will be necessary. Studies involving individual enzymatic reactions within a given community would be a later important extension of the program, since extracellular microbial enzymes may prove to be major catalysts in sediment biochemistry.

At the moment, however, the major barrier to such a program is the lack of adequate methods for studying mixed cultures. Most microbiologists, trained to think only in terms of pure cultures, will have to acquire a new set of concepts to understand the changes occurring in these much more complex systems. Mixed-culture studies might provide the details of microbial activity in a given ecological situation, at least for the molecules of major importance—sugars, amino acids, fatty acids, and their derivatives.

Recognition of a need for methods of mixed-culture study is certainly not new; this need has been discussed in detail by Winogradsky, and by many others since his time. In recent years, several Russian workers have presented excellent reviews on this subject, with reference only to soils, however. The need for learning how to study mixed cultures has been particularly well stated in papers by Khudyakov (1954) and Mishustin (1954). However, practically nothing has ever been done to develop methods for the specific study of mixed-culture systems in soil or sediment. To be sure, mixed cultures have been used in some research projects, but the results have not been interpreted in terms of the contribution of each component microbe or molecule.

PREVIOUS WORK

Microbiology

A fair amount of data is available on bacterial populations in the uppermost portion of marine sediments, but there are few detailed reports dealing with analyses of long cores. ZoBell and Anderson (1936) and ZoBell (1938) reported that, in general, bacterial populations decreased with increasing depth of sediments. ZoBell (1938) presented a general curve showing a rapid decrease of organisms from 10^7–10^8/g in the upper few inches of a core, to about 100/g at 3 ft. These data came from cores taken in the channel island area off southern California.

In 1942, ZoBell reported results obtained from additional cores taken there. These results showed aerobic bacteria decreasing from as high as $38 \cdot 10^6$/g at the top to about 150/g at about 8 ft.

Rittenberg (1940) also analyzed several cores from the California offshore basins. He reported three general types of sediment with regard to bacterial population, and classified them as high, medium, and low population groups. All followed a similar pattern of rapid population decrease below the water-sediment interface; however, those in the intermediate class showed unexplained increases at certain points below 2 ft. All his analyses were on cores 4 to 7 ft long except one (the one with the most bacteria) which was 11 ft in length. A core of pelagic sediment showed an almost constant population of around 10,000/g for its 7 ft length.

All the figures given above are for organisms growing aerobically under laboratory conditions. This does not necessarily mean that aerobic conditions exist in all these sediments in nature. Actually, most of the so-called aerobes are facultative, and grow very well anaerobically.

Interpretation of these reports is difficult since no geological or physical data are given to help explain the variations. We believe that environment of deposition is the most important variable in this kind of study—that is, that there is no such thing as a "typical" marine core.

The data of Oppenheimer (1952) for the Mississippi Delta and for certain Gulf Coast bays are similar to those of the earlier reports. Although wide variations also exist in Oppenheimer's data, the deltaic muds in general showed a slower decrease in population than did the bay sediments. Again, lack of geological information on the individual cores makes interpretation difficult.

Microbiological work on sediments from many large bodies of fresh and salt water in Europe and Asia has also been reported (see for instance Lambina, 1957; Zhukova, 1955; Zavarzina, 1955), but either the samples have been taken over a wide area at the surface of the sediment, or they have been single cores only a few feet in length. Environmental control is therefore lacking.

Biochemistry

General biochemical analysis of marine muds has been reported only a few times, again mostly by Russian workers. While many others have analyzed in great detail for particular fractions or compounds, the researches of Shabarova (1954, 1955a, 1955b) and Rodionova (1951), are the best examples known to us where an attempt was made to understand the total biochemistry of a sediment.

The comments made above in reference to sediment microflora apply here. Samples for biochemical analysis have usually been taken in widely separated spots, and almost entirely from the surface of the sediment.

RESULTS

Our work is, in general, following the plan just outlined. We are attempting to gain as much environmental control as possible before beginning more detailed studies. Sediments have been obtained from well-defined environments, and relative populations of a number of bacterial physiological types have been determined by standard extinction dilution methods. To obtain the greatest number of heterotrophic bacterial types from a given sample, a variety of enrichment cultures, for aerobic, anaerobic, saccharolytic, proteolytic, lipolytic, and other types of organisms, has proven to be best. Such enrichments have been used for the same type of work by ZoBell (1946) and others. Our media, however, differ slightly for some of the cultures. For each sample, pH and Eh are determined, and geological descriptions and analyses are recorded. Also, analyses are made for the major biochemical fractions. Correlation of these chemical data with microbial physiology of the strata sampled is the first objective.

We have studied carbonate muds in Florida Bay and in the Gulf of Batabano south of Cuba; deltaic deposits in the delta of the Orinoco River in northeast Venezuela; evaporites from the Bocana de Virilla in Peru; and samples from the Cariaco Trench off northern Venezuela, and from the Gulf of California. Sediments of the Norwegian Trough in the North Sea are presently being studied.

Microbiology

Our hypothesis that geography and geology have an effect on the distribution of types of microorganisms has so far proven true. Even if what appears to be the same organism (morphologically) is found in more than one environment, or at different depths in the same environment, it is usually different in its metabolic abilities.

A striking difference has been noted between the microflora of carbonate muds and deltaic deposits (Fig. 1). In the carbonates we have studied in Florida and Cuba, we have rarely obtained heterotrophic organisms from samples taken below 3 ft. In the clays of the Orinoco Delta, however, the populations remain high well past this point. That this may be a common feature of deltas is indicated by Oppenheimer's (1952) results in the Mississippi Delta. In the Orinoco Delta we have found viable bacteria to a depth of about 150 ft, although 80% of the isolates were from above 50 ft. A reason for larger populations in deltaic sediments than in carbonates might be a greater quantity of organic material present in the delta; on the other hand, the extremely reducing character of the carbonates studied and their high H_2S content may limit bacterial growth. The average Eh of 11 cores from Florida Bay and the Gulf of Batabano is about −200 mv, while most sediments examined in the Orinoco Delta have a positive Eh even at great depth.

Two cores about 4 ft in length from Florida Bay are interesting examples. Core 1 was taken on the surface of an algal mat on North Crane Key. After penetrating the mat and some algal debris, it entered fine lime mud, then a section of mangrove peat, and finally more mud. The pH and Eh of the samples in this core

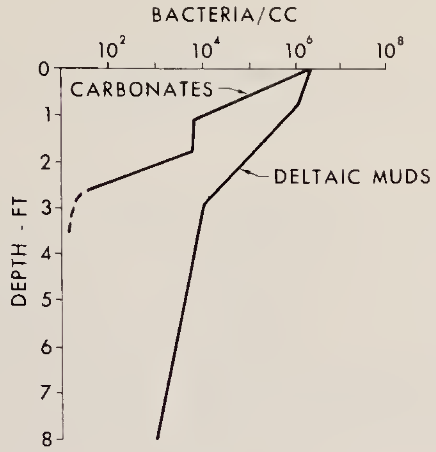

Fig. 1. Relative total bacterial populations in carbonates and deltaic muds.

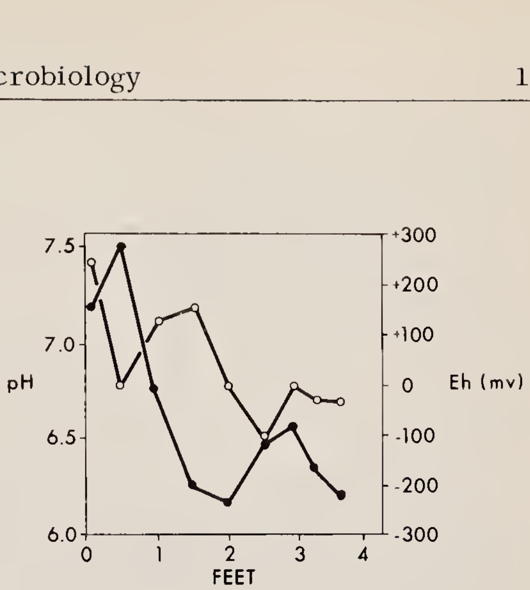

Fig. 2. Changes in pH and Eh with depth (Florida Bay Core 1). Open circles, pH; solid circles, Eh.

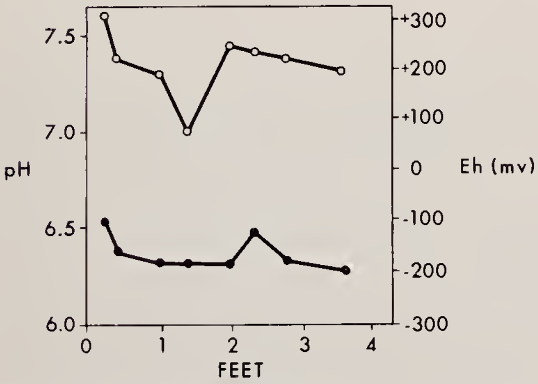

Fig. 3. Changes in pH and Eh with depth (Florida Bay Core 2). Open circles, pH; solid circles, Eh.

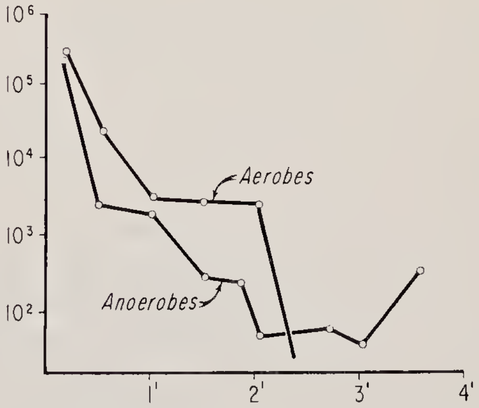

Fig. 4. Changes with depth in relative populations of bacteria growing in aerobic and anaerobic enrichment cultures (Florida Bay Core 1).

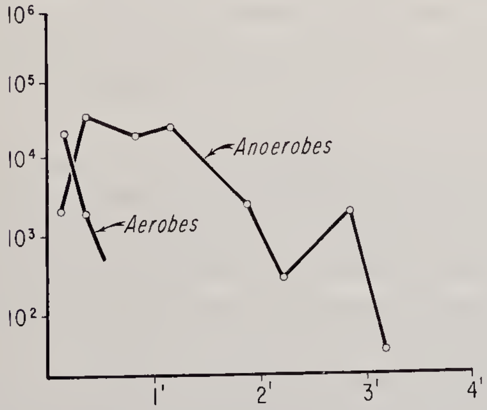

Fig. 5. Changes with depth in relative populations of bacteria growing in aerobic and anaerobic enrichment cultures (Florida Bay Core 2).

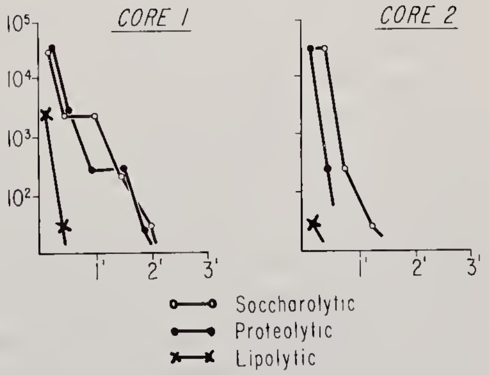

Fig. 6. Changes with depth in relative populations of bacteria growing in starch, gelatin, and tributyrin enrichment cultures (Florida Bay).

are shown in Fig. 2. It can be seen that the Eh becomes negative about one foot from the surface.

The second core was taken from sediment covered by about 3 ft of water. The pH and Eh curves for this core (Fig. 3) show that the sediment is strongly reducing even at the surface.

Figure 4 shows the relative populations of organisms growing in aerobic and anaerobic enrichment cultures from Core 1. The aerobes failed to grow below about 2 ft, while the anaerobes persisted to about 3½ ft but were not detected at lower levels.

The populations from Core 2, shown in Fig. 5, are quite different; the aerobes disappear in only a few inches but the anaerobes persist until just below 3 ft.

This is a rather simple but striking example of the effect of physical conditions on the microbial populations.

Figure 6 shows the relative populations of those organisms hydrolyzing starch, gelatin, and tributyrin. It is interesting that in Core 2, the more reducing environment, saccharolytic organisms persisted longer than proteolytic organisms, but in Core 1 they declined at about the same rate. The lipolytic bacteria, on the other hand, were present only in very low numbers in Core 2 and disappeared several inches earlier in this core than in Core 1.

Sulfate-reducing bacteria were present only in the upper 6 in. of each core, about ten times as many in Core 2 than in Core 1. However, the deeper sediments contained much H_2S, the origin of which is not explained by these sulfate-reducer counts. It is probable that proteolytic pseudomonads found at lower levels contribute the bulk of the sulfide.

Preliminary results show that the organisms isolated from these cores, taken as a group, also show different metabolic patterns. The ability to ferment certain mono- and disaccharides is especially marked in the organisms from Core 2. From 90 to 100% of the total organisms in this core show this ability, compared with only 60 to 75% of those in Core 1. The individual cultures from Core 2 are also more actively proteolytic than those from Core 1.

Figure 7B shows the distribution of bacteria in Orinoco Delta muds. The curves represent averages from three 150-ft holes. The same decrease in populations found in other marine sediments also appears here, but the anaerobic peaks at 36 and 100 ft seemed anomalous to us at first. However, analysis of gas in the muds showed not only a decreasing trend in CO_2 (as one would expect), but also high methane concentrations which coincided with the high populations of anaerobic bacteria (Fig. 7A).

An interesting auxiliary piece of data on these sediments is that fungi, some of which are quite different from common fungal surface contaminants, have been found in many of the deep cores (Roth, 1959). The presence of these forms in a habitat usually considered extremely unfavorable poses a fascinating problem in microbial ecology. Such a problem has been mentioned, for fungi in soils, by Burges and Fenton (1953) and Hawker (1957).

The pH and Eh of the deltaic sediments fall generally in the "connate water" and "heterotrophic bacteria" classes of Baas Becking, Kaplan, and Moore (1960). The latter environment they classify as "similar to many anaerobic sedimentary environments in nature where large quantities of organic material accumulate."

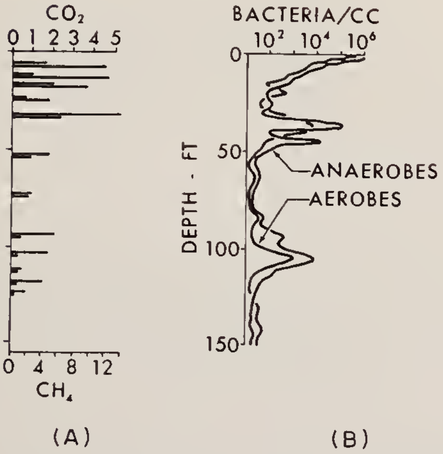

Fig. 7. A) Gas in sediment samples at various depths. Upper bar at each depth represents CH_4; lower bar represents CO_2. Amount of each gas given in cubic centimeters per 100-ml wet sample (Orinoco Delta). B) Changes with depth in relative populations of bacteria growing in aerobic and anaerobic enrichment cultures (Orinoco Delta).

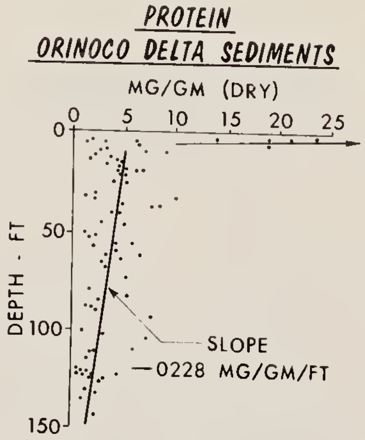

Fig. 8. Results of analyses for protein.

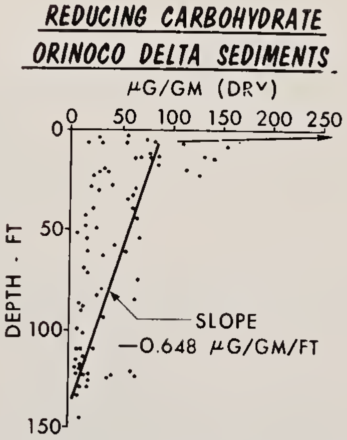

Fig. 9. Results of analyses for reducing carbohydrate.

Biochemistry

Since all organic material, no matter what its source, contains protein, carbohydrate, and lipid fractions, we hoped that data on these could be used to define generally the microbial nutrition level at any given depth.

Shown in Fig. 8 are the results of analyses for protein in these sediments. After 24-hr cold digestion of the sediment sample in 8% sodium hydroxide followed by filtration and dilution, protein was estimated by spectrophotometric measurement of the color developed with the Folin reagent. This method was adapted from Krey's (1951) method for analyzing protein in marine plankton. The concentration decreases rapidly with depth to just less than 10 ft, from which point it decreases much more slowly. A least-squares line through the points below 10 ft has a slope that corresponds to a decrease of 22.8 μg/g dry weight of sediment per foot. The correlation coefficient of -0.435 is significant at the 99.9% level.

Figure 9 shows the same type of curve for reducing sugars as a reasonable estimate of available carbohydrate. These were measured by extracting the mud

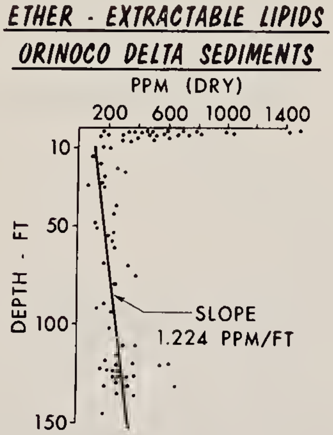

Fig. 10. Results of analyses for ether-extractable lipids.

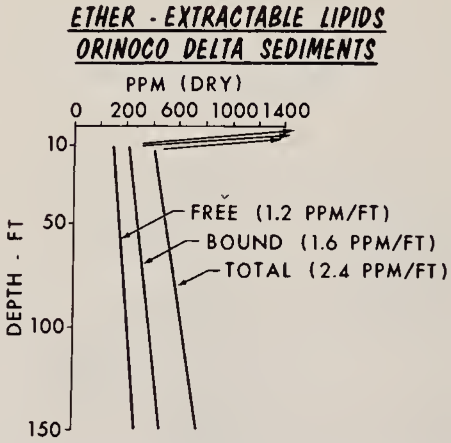

Fig. 11. Regression lines through data points (not shown) for free, bound, and total ether-extractable lipids.

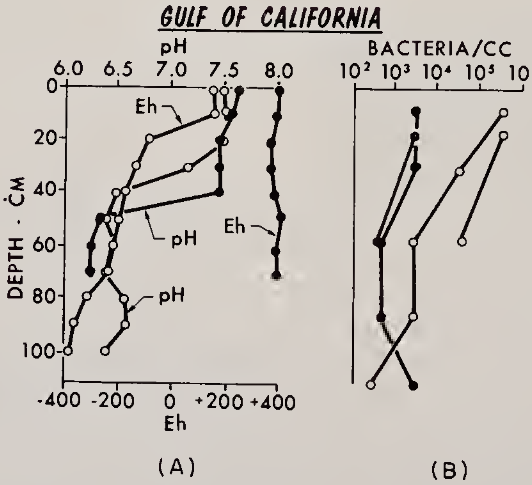

Fig. 12. A) Changes with depth in pH and Eh of two cores from Gulf of California sediments. Solid circles, Core 1. Open circles, Core 2. B) Changes with depth in relative populations of bacteria growing in aerobic and anaerobic enrichment cultures. Core 1, 2 samples. Core 2, 5 samples. Open circles are aerobes and solid circles are anaerobes.

with warm 80% ethanol, concentrating the extract by evaporation, diluting with water, and using this solution for reduction of ferricyanide. The color that developed by adding ferric ion was measured in a spectrophotometer. The carbohydrates also show a rapid decrease, reaching the change of slope a few feet earlier than the protein. The remainder of the curve shows a more erratic behavior than the protein curve but also shows a gradual decrease—in this case, 0.648 μg/g-ft. The correlation coefficient of −0.468 for this line below 10 ft is also significant at the 99.9% level.

Figure 10 shows the same type of curve for ether-extractable lipids such as glycerides, fatty acids and esters, porphyrins, carotenoids, and hydrocarbons. The dry sediment was extracted in a standard Soxhlet apparatus. Here again, we see a rapid decrease to about 10 ft, but now a slight *increase* occurs, amounting to 1.224 ppm per foot of sediment. The *positive* correlation coefficient of +0.434 is significant at the 99% level. This correlation is independent of lithology.

The scattered distribution of points in these curves is most likely due to different amounts of organic material deposited at various times in the history of the sediments. However, the scatter is not so great when one considers that the vertical axis of the graphs is greatly reduced compared with the horizontal axis, which shows extremely small amounts of protein, sugar, and lipid.

Several workers, in this country and abroad, have noted that an additional amount of lipid is extractable after treatment of the once-extracted sample with 0.1 N HCl. This is the so-called "bound lipid," which often proves to be a large part of the total. Our Orinoco Delta samples were treated in this manner, and the data analyzed as before. Figure 11 shows the correlations obtained. The "bound" fraction increases at a slightly greater rate than the "free lipid," showing a slope corresponding to an increase of 1.61 ppm/ft. When the two sets of data are added and the sums at each level plotted, the increase is 2.4 ppm/ft. The correlation coefficients for "bound" and "total" lipid are significant at the 97% and 98% levels, respectively.

At the present time, we are not prepared to state with certainty the source of this added lipid in the deltaic mud. However, one intriguing possibility is that it represents microbial metabolic products formed at the expense of the other fractions.

We have studied certain other environments in less detail. Samples from the Cariaco Trench were made available to us by Dr. John Zeigler of Woods Hole Oceanographic Institution. Our biochemical results on cores from the Trench are shown in Table 1. Here the pattern is essentially the same as in the Delta. The

Table 1. Biochemical Analyses of Cariaco Trench Samples

	Depth (ft)	Protein (mg/gm)	Lipids (ppm)			
			Free	Bound	Total	% Bound
Core 1						
	3	22.92	521	31	552	5.6
	13	9.44	580	310	890	34.7
	19	8.33	709	227	936	21.9
	27	6.70	422	41	463	8.9
Core 2						
	½	10.00	745	168	913	18.4
	7	5.20	548	138	686	20.1
	11	0.85	93	78	171	45.6
	23	0.89	189	196	385	50.9

proteins decrease with depth; the free and total lipids increase below 10 ft, except in the last sample of Core 1. Of course, the analysis of only two cores cannot be used to indicate trends. The last two samples in the second core are from an oxidized sediment, probably Pleistocene, into which the core penetrated. The increase in lipid with depth is exceptional here. Also, it is interesting to note the increase, with depth, in the *per cent* of total lipid that is in the "bound" state.

Data have also been obtained from two cores from the Gulf of California. These cores were frozen immediately after collection and were sent to us in that condition through the courtesy of Dr. T. H. van Andel of Scripps Institution of Oceanography. The cores were taken in water depths of 832 and 931 fathoms, respectively. The bacterial populations for these cores, and the Eh and pH values obtained by the Scripps personnel aboard ship, are shown in Fig. 12. The biochemical data we have obtained appears in Table 2.

Table 2. Biochemical Analyses of Gulf of California Samples

	Depth (ft)	Protein (mg/gm)	Sugars (μg/gm)	Lipids (ppm)			
				Free	Bound	Total	% Bound
Core 1							
	1	10.17	464	1101	204	1305	15.6
	2	6.39	311	322	206	528	39.0
Core 2							
	1	17.02	554	1306	340	1646	20.7
	3	11.72	264	1298	467	1765	26.5
	4	13.63	345	1016	527	1543	34.2

Results on these cores indicate that this environment is richer in organic matter than any other we have studied. The analyses follow the same pattern as those from the other environments, but at a much higher level. The interesting increase with depth in the per cent of "bound" lipids appears here also. This increase has not been noted in the deeper sediments from the Orinoco Delta.

Detailed bacteriological work has begun with the preservation by freeze drying of over 1000 bacterial isolates, which include aerobes, facultative organisms, and obligate anaerobes from the locations discussed, and from others; and these are presently being studied to determine whether there are any simple biochemical characteristics that can be used to categorize organisms from specific environments. The data so far are incomplete, but do show great promise for such classification. For instance, the carbohydrate fermentation mechanisms and patterns for organisms from the Orinoco Delta differ greatly from those for any other area we have studied; the organisms from Peruvian evaporites are more actively lipolytic than those from other environments; and, compared with organisms from other sediments, a much greater percentage of carbonate mud bacteria reduce nitrate in the laboratory, even though the supply of nitrate in their natural environment is practically zero. These, and other interesting results are being catalogued, and data from other environments will be added as they are obtained. We expect that when this fundamental work is completed and published, a large collection of rather specific marine microflora will be available to workers in marine microbiology for further studies.

The complex ecological studies that are necessary are in the planning stages. No data are yet available.

As mentioned before, our purpose here has been to present a working philosophy of research on the microbiology and biochemistry of recent marine sediments, and to stimulate the interest of others in the research needs in this field.

The results presented are admittedly preliminary, although they do show the type of data that can be obtained in such a program as we have outlined. Many interpretations might be made of such data, both academically and industrially, and depending on the viewpoint of the observer. However we would rather reserve such postulations until the experimental part of the program progresses further. The problem of the activity of heterotrophic microbes in sediments is of such broad scope, involves so many scientific disciplines, and explores fundamental processes so little understood that we feel it is best not to sidetrack the program in an immediate search for practicality before those factors which control the total picture are at least partially understood.

ACKNOWLEDGMENTS

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MICROBIAL OXIDATION OF n-PARAFFINIC HYDROCARBONS

R. L. Raymond*
Socony Mobil Oil Co., Inc., Field Research Laboratory, Dallas, Texas

Beerstecher in his book *Petroleum Microbiology* made the following observation: "Petroleum fermentation offers tremendous possiblities for the development of new products. Microorganisms might be used to make many chemicals from petroleum, or they might be used in unit processes in the petrochemical industry. High grade edible fat and protein might also be derived from petroleum through microbial action. In addition, various drugs might be obtained in this manner. Despite the many attractive possibilities in this area, there appears to be at present very little study of the field, and many basic data are needed before the horizons of microbiological petrochemistry can be accurately visualized."

Today, several years later, the use of hydrocarbons as substrates for microorganisms for the production of industrial chemicals is still not realized. Fortunately, however, some basic studies are being done in our universities and elsewhere so that petroleum fermentation may become a reality. With the interest that is being shown in elucidating the mechanism of n-alkane oxidation by microorganisms it seems appropriate to review some of the more recent literature pertaining to methods of isolation, description of microorganisms, and the means of culturing hydrocarbon oxidizers used in these studies. (Leadbetter and Foster, 1960; Stewart *et al.*, 1959; Thijsse and Van Der Linden, 1958).

Webley and De Kock cultured *Nocardia opaca* on glucose for oxidation studies of alkanes because of the difficulty of separating the cells from the hydrocarbon. In a later paper Webley states that this microbe grows well on n-dodecane, n-tetradecane, n-hexadecane, and n-octadecane. He found it grew poorly on heptane, octane, and nonane but could not really assess growth on any of the hydrocarbons tested due to the nature of the substrate. Treccani and Canonica were able to grow a corynebacterium isolated from decomposing organic matter on several aliphatics from C_{11} to C_{28}. Treccani *et al.* isolated three additional microbes, an achromobacter, a nocardia, and a mycobacterium by soil enrichment with pentane and hexane. They obtained pure cultures by plating on nutrient agar. To test growth on the light hydrocarbons up to C_8 the cultures were placed in glass containers and allowed to grow on the vapors. The liquid hydrocarbons were first adsorbed on filter paper and placed in the lid of the Petri dish. All of the cultures tested with the exception of the achromobacter could grow on several n-alkanes from C_3 to C_{28}. The achromobacter utilized C_3 through C_{12}. After Warburg respirometer studies, they concluded that the metabolism of the hydrocarbons was the same (after the initial oxidative step) as the saturated fatty acids, supporting the mechanism of β-oxidation.

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*Present address: Sun Oil Co., Marcus Hook, Pennsylvania.

Konovaltschikoff-Mazoyer and Senez found eleven strains of *Pseudomonas* in soil and water, probably contaminated by petroleum products, capable of using C_3 to C_{16} aliphatic paraffins as the sole source of carbon. For their enrichments they used 1 g soil in 1 L mineral-salts medium with 1% hydrocarbon added. They obtained pure cultures by streaking on mineral agar and incubating under heptane vapors. They reported 97 mg of cells of one strain from 1 L of medium containing 1% n-heptane. Later Senez and Konovaltschikoff-Mazoyer grew *Pseudomonas aeruginosa* in 2-L flasks containing 1 L mineral-salts medium and 10 g n-heptane. After six days they reported the formation of 1.08 mM of volatile fatty acids which they identified by paper chromatography. They did not report on the amount of cells produced or the quantity of substrate utilized.

Harris isolated a micrococcus from an asphalt-soil interface which grew well on a number of aliphatic hydrocarbons, but no account was given for method of isolation or determination of growth. Complete oxidation of the hydrocarbons to CO_2 and water was indicated by oxygen uptake and respiratory-quotient studies using glucose-grown cells.

Azoulay and Senez grew cells of *Pseudomonas aeruginosa* on nutrient medium to which they added 5 ml/L of n-heptane. They obtained 2 g of cells from 10 L of medium in 36 hr at 32 C. The cells were used to demonstrate dehydrogenation of several long-chain n-paraffins. They used pyocyanin as an indicator for the dehydrogenation.

Stewart *et al.* and Stewart and Kallio, while studying ester formation by a Gram-negative coccus, set up large scale cultures in 3 L Fernbach flasks containing 1 L mineral-salts medium. Hydrocarbons were added (1 vol. %) and cultures incubated up to 210 hr on a gyratory shaker (230 rpm). They found that decane, dodecane, tetradecane, hexadecane, and octadecane could all be used as substrates for the microorganism, but they reported their yields of cells in ml of packed cells per 6.5 ml of medium. Two and one half grams of ester (calculated as cetyl palmitate) was reported to have been formed from 7.73 g of hexadecane.

Hoerburger in an industrial laboratory grew *Candida tropicalis* in 450 ml mineral salts containing 1.7% of a hydrocarbon "cut" (b.p. 230–290 C). With high aeration (400 L/hr of air) he was able to increase the amount of yeast cells from 1 g to 2.65 g in 22 hr. Because of this high aeration requirement and the variable composition of the cut used, he concluded that the production of protein by this method would not be industrially feasible.

The soil enrichment technique seems by far the most widely used method described in the literature of obtaining isolates from nature. The advantages and limitations of this method have been discussed recently by Leadbetter and Foster for methane-utilizing bacteria and apply equally well to other n-alkane-utilizing microbes. Factors such as pH, temperature, and source of inoculum determine to a great extent the types of microorganisms which are isolated. While the numbers of microbes are greatly enhanced by gas leaks, oil seeps, and contamination by petroleum products, many bacteria, yeasts, and molds can be isolated from soils without such a history. In our laboratory we used the sprinkled-soil plate method described by Davis *et al.* and Leadbetter and Foster and observed several hundred colonies of nocardiae, corynebacteria, streptomyces and molds on a single plate from 0.1 g garden soil, with hexadecane as the carbon source.

It is apparent that although there are many reports of the utilization of a wide range of n-alkanes by many kinds of microbes there are still little if any data available on the yield and composition of cells and products. It is the purpose of the balance of this paper to discuss experimental results obtained in our laboratory using 4 L fermentors for the oxidation of n-alkanes by a nocardia and by a yeast.

MICROORGANISMS

The nocardia was isolated from an oilfield soil in one of the surveys described by Davis *et al.* A description of this microbe has been given by Raymond and Davis. Figure 1 shows a phase-contrast photograph of a 24-hr culture grown on a mineral agar plate with hexadecane vapors as the only source of carbon. This nocardia is representative of many pigmented strains which we have observed in many different types of soils both on and off oilfields.

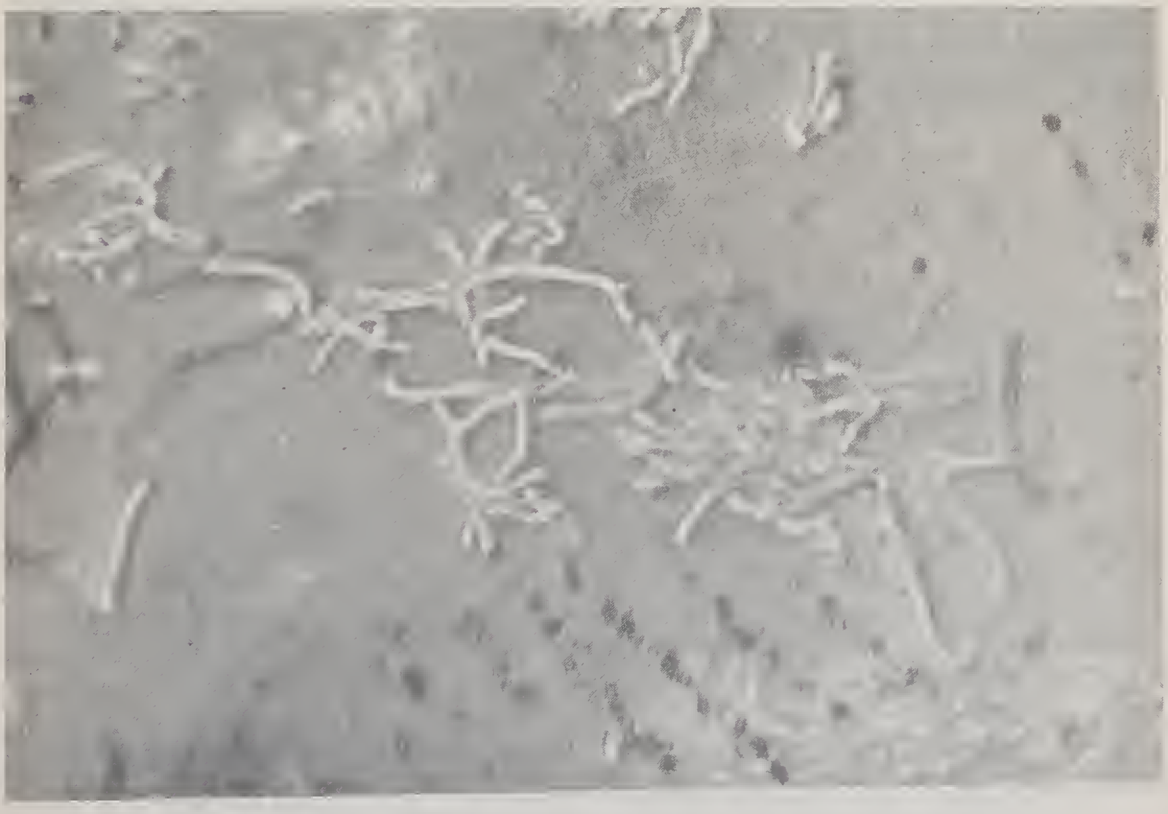

Fig. 1. Cells of a *Nocardia.* sp. (24 hr) grown on hexadecane vapors. Phase-contrast photomicrograph.

The yeast was isolated from topsoil near Dallas, Texas by the enrichment technique. One gram of soil was placed in 50 ml of Czapek's mineral medium in which 5% n-octadecane was substituted for sugar. The pH was maintained around 4.0 and the system incubated at 30 C on a gyratory shaker. After several days, microscopic examination revealed small yeast-like organisms. These were isolated on glucose – peptone – yeast extract agar and transferred to the modified Czapek's medium using octadecane as the only source of carbon. Young cultures

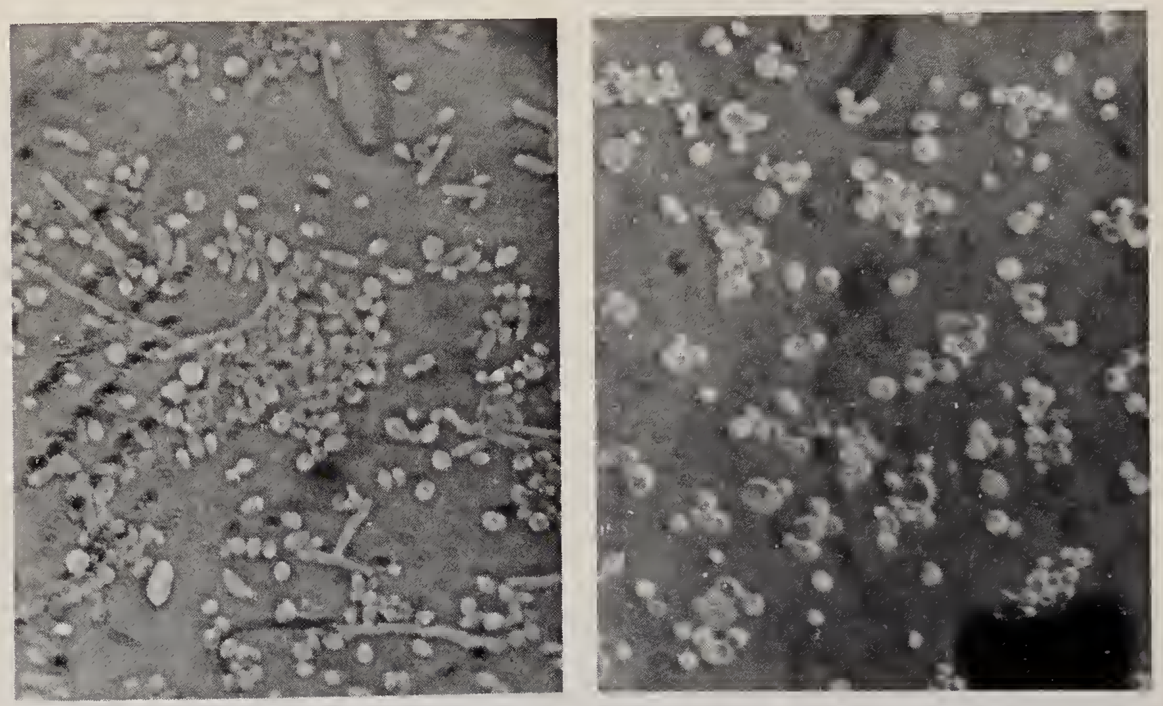

Fig. 2. Yeast cells grown on n-hexadecane. Phase-contrast photomicrographs.

of this microbe form long filaments or a pseudomycelium in stationary nutrient and n-alkane media. In shake systems or fermentors it looks like a budding yeast. Figure 2 is a phase-contrast photograph of both forms. In the fermentors the cells are about 2.0 × 4.0 μ. The colonies are smooth, convex, soft, and cream-colored on nutrient agar plates. Growth on n-octadecane is much better with nitrate than ammonium sulfate as a nitrogen source. This microbe has been tentatively identified as belonging to the genus *Candida*.

OXIDATION SYSTEMS

The fermentors used have been described by Raymond and Davis. The size of the baffles, impeller, and other dimensions were designed very close to those recommended by Finn for maximum turbulence. Figure 3 shows a bank of these systems used to study oxidation of hydrocarbons. The speed of agitation has a large effect on the growth rate of cultures on hexadecane and octadecane. It was found that an optimal rate could be obtained at 1600 to 1750 rpm. At this speed with 2 L of media in each system the substrate was well emulsified into very small globules with very little tendency to float to the surface. This agitation effect was demonstrated with the nocardia culture, where under similar conditions except for rate of agitation, 6.4 and 8.3 g of cells were produced in the same length of time at 1150 and 1725 rpm, respectively. This vigorous agitation has the added effect of reducing the amount of air that had to be provided through the sparger. A flow of 100 to 200 ml/min of air was used to provide a slight positive pressure inside the fermentors to reduce the chances of contamination.

It was observed that several factors affected growth and composition of nocardia and yeast cells in the fermentors. First the concentration of substrate relative to the amount of inoculum was very critical for the nocardia. If the

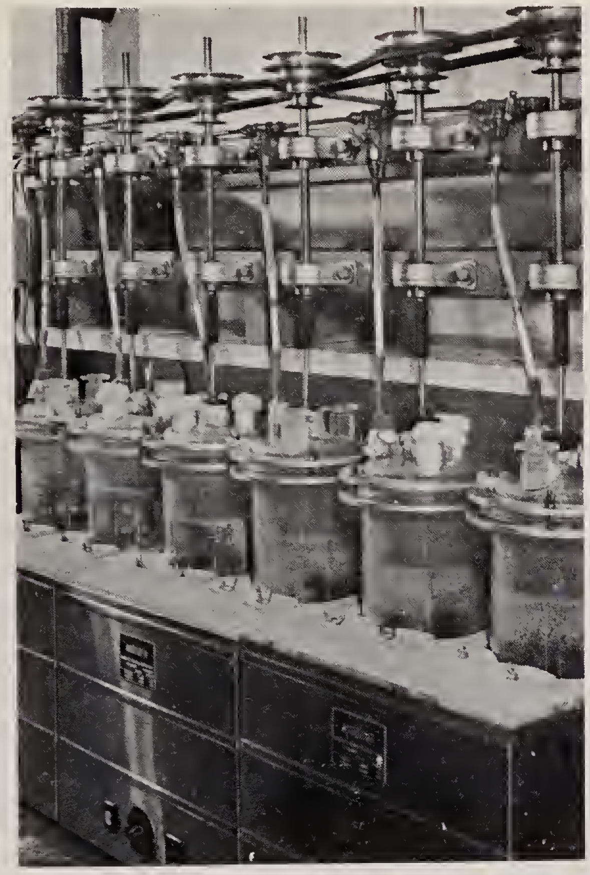

Fig. 3. 4-L fermentors used to study oxidation of n-alkanes by microorganisms.

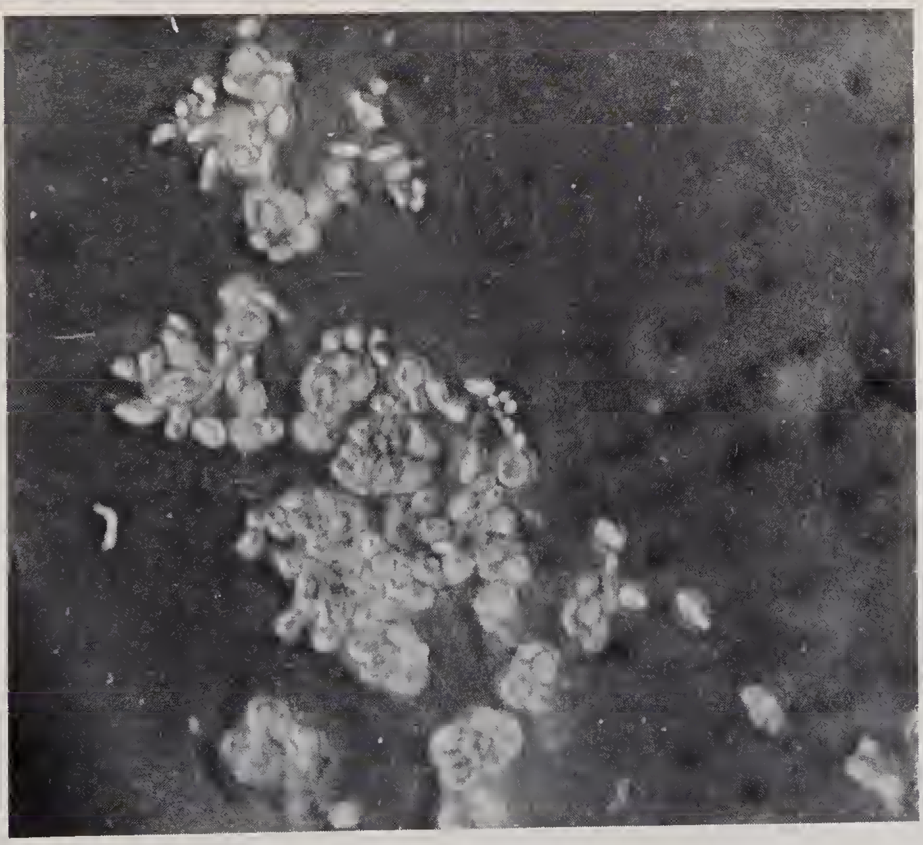

Fig. 4. Cells of a *Nocardia* sp. containing 78% lipid. Grown on n-octadecane with asparagine as N source. Phase-contrast photomicrograph.

ratio of n-octadecane to cells was 20 : 1 or greater no growth occurred. Under similar circumstances a ratio of 7 : 1 yielded excellent growth. Thus it was necessary when starting with a small inoculum to add small quantities of substrate early in the fermentation. Although this point was not specifically tested for the yeast in the fermentors, tests in 250-ml indented shake systems indicated that 5% octadecane had no adverse effects. The probable cause of this difference can be observed microscopically. The nocardia cells become so firmly adsorbed to the substrate that cells are not visible under phase contrast unless a small amount of some dye such as rose bengal is first added to the suspension. The yeast cells are readily seen without such treatment and do not appear to be adsorbed very firmly to the substrate. Evidently in the instance of high substrate-to-cell ratio the nocardia cells are so completely surrounded by substrate that other nutrients are not available. This adsorption difference between the nocardia and yeast is also observed when the cells are centrifuged and excess substrate remains. Most of the yeast cells are thrown down while the nocardia cells remain trapped in the floating oil layer and have to be recovered by washing with petroleum ether. A schedule of addition of substrate which was quite successful for the nocardia and yeast are shown in Tables 1 and II.

Table 1. Oxidation of n-Octadecane by a Nocardia in 4-L Fermentors

Substrate added (g)*	80.00
Substrate remaining (g)	28.00
Cells produced (g)	49.80
Lipid (%)	59.8
Polymer (g)	2.8

*Urea and n-octadecane were added to 2 L of mineral-salt medium (0.6% Na_2HPO_4, 0.4% KH_2PO_4, 0.08% $MgSO_4$) in the following manner: initial, 0.6 g urea and 8.0 g n-octadecane; 17 hr, 0.3 and 8.0; 40 hr, 0.6 and 16.0; 48 hr, 1.5 and 48.0. Length of incubation 112 hr, temperature 30C, impeller 1600 rpm, and air 200 ml/min.

Varying the type and concentration of nitrogen affected both the cell yields and lipid production. The results are similar to those observed for sugars for maximum lipid production by other microorganisms, i.e., high C : N ratios favor lipid synthesis, and low C : N ratios increase protein synthesis. The nocardia has been studied more thoroughly than the yeast and these effects are demonstrated for this microbe in Tables III and IV. Figure 4 is a photograph of the nocardia cells containing 78% lipid as the result of growing on n-octadecane and 0.1% asparagine.

Lowering the oxygen tension in the fermentors did not result in any changes in product formation by nocardia but it markedly changed the composition and yield of cells. The results of this study are shown in Table V. It should be noted that the yield of cells decreases as the oxygen concentration is lowered. It is possible that there was an increase in a water-soluble polymer, later shown to be produced by this microorganism when growing on hydrocarbons. This polymer

Table 2. Oxidation of n-Octadecane by a Yeast in 4-L Fermentors

	0.2% NH_4NO_3	0.1% NH_4NO_3	0.1% NH_4NO_3
Substrate added (g)*	28.0	12.8	9.6
Substrate remaining (g)	10.1	1.4	0.4
Cells produced (g)	12.2	8.5	7.5
Lipid (%)	6.7	-	18.2
Liquor analyses			
Ether extract (g)	0.54	0.45	0.40
Volatile acids (meq)	2.5	0.0	0.0

*n-Octadecane added to 2 L of mineral-salts medium (0.01% Na_2CO_3, 0.001% $CaCl_2$, 0.002% $MnSO_4$, 0.001% $FeSO_4$, 0.3% Na_2HPO , 0.02% $MgSO_4$, 0.2% KH_2PO_4) in the following manner:

initial	4.0 g	-	1.6 g
2 hr	-	0.8 g	-
7	-	4.0	-
16	8.0	-	-
20	16.0	-	4.0
24	-	8.0	-
26	-	-	4.0
Length of incubation (hr). . . .	96	96	41

Table 3. Effect of Nitrogen Sources on Nocardial Cell Yield and Lipid Formation

(Substrate, 824 mg n-octadecane)

Nitrogen Source	N in cpd. in %	n-Octadecane utilized in mg	Dried cell wt. in mg	Conversion to cells in %	Cell lipid in %	Final* pH
$(NH_4)_2SO_4$ (0.1%)	21	606	445	74	72	6.0
$(NH_4)_2SO_4$ (0.3%)	21	293	224	83	43	5.0
NH_4NO_3 (0.3%)	35	293	207	70	46	5.0
KNO_3 (0.1%)	14	394	252	64	70	8.0
KNO_3 (0.3%)	14	795	530	67	65	8.1
Urea (0.1%)	47	808	603	75	64	7.0
Urea (0.3%)	47	772	522	68	27	7.2
Asparagine (0.1%)	21	512	369	72	78	6.9
Asparagine (0.3%)	21	791	586	74	69	6.0

*The initial pH was buffered at neutrality with the 0.5% phosphate salts in the regular mineral-salts medium. Incubation, 144 hr at 30° C in 100 ml medium in 250 ml bottles agitated on gyratory shaker.

has not been identified but is produced in good yields under some circumstances. This was not known at the time of these experiments on oxygen concentration.

Table 4. Effect of Nitrogen Concentration on Oxidation of n-Octadecane by Nocardia in 4-L Fermentors*

Octadecane added (g)	32.000	80.000	100.000
Octadecane recovered (g)	11.620	29.181	33.030
Cells recovered (g)	13.800	43.286	57.394
Lipid in cells (%)	65.2	55.9	50.1
Liquor analyses			
Ether extract (g)	0.188	0.310	0.614
Carbon analysis (g)	-	3.290†	3.510†
Volatile acids (meq)	0.0	3.7	7.9
Nitrogen added (g)	0.42 $(NH_4)_2SO_4$	2.2 $(NH_4)SO_4$	3.5 Urea
Residual nitrogen	-	0.437	0.475

*Impeller speed 1725 rpm, pH 7.1, temperature 30 C, air flow 60 to 150 ml/min. Mineral medium had the following composition: 0.01% Na_2CO_3, 0.001% $CaCl_2$, 0.002% $MnSO_4$, 0.02% $MgSO_4$, 0.001% $FeSO_4$, 0.3% Na_2HPO_4, 0.2% KH_2PO_4. Incubation time 120 hr.

†Capsular slime or polymer.

Table 5. Oxidation of n-Octadecane by Nocardia Under Varying Oxygen Concentrations in 4-L Fermentors*

	20% O_2	10% O_2	2% O_2
Substrate added (g)	10.400	10.400	10.400
Substrate recovered (g)	0.293	0.363	4.707
Cells (g)	8.519	6.612	3.073
Lipid in cells (%)	56.0	73.0	21.0
Acetone-insoluble lipid in cells (%)	21.6	7.9	2.8
Liquor analyses			
Ether extract (g)	0.198	0.289	0.093
Volatile acids	0	0	0
Air Flow (ml/min)			
0 to 24 hr	100	100	100
24 to 48 hr	200	180†	260‡

*Physical conditions: 3 L of mineral medium of following composition: 0.01% Na_2CO_3, 0.001% $CaCl_2$, 0.002% $MnSO_4$, 0.02% $MgSO_4$, 0.001% $FeSO_4$, 0.075% Na_2HPO_4, 0.05% KH_2PO_4, 0.1% $(NH_4)_2SO_4$. Impeller speed 1725 rpm, temperature 30 C,. pH 7.1.

†10% O_2 + 90% N_2.

‡2% O_2 + 98% N_2.

When the nocardia cells were extracted with chloroform-methanol (2 : 1) they were found to be very high in lipid in those systems containing hexadecane or octadecane as the substrate. This effect of length of the hydrocarbon chain on the lipid is shown in Table IV and VI. Glucose-grown nocardia contained from 16-28% lipid. This high lipid content peculiar to hexadecane- and octadecane-grown cells was due largely to waxes which make up to 40% of the lipid. The percentage of the total cell weight of these waxes is listed as the acetone-

Table 6. Oxidation of n-Hexane, n-Tridecane and n-Hexadecane by Nocardia in 4-L Fermentors*

	n-Hexane	n-Tridecane	n-Hexadecane
Substrate added (g)	55.000	21.000	40.196
Substrate remaining †	-	-	-
Cell (g)	4.399	7.52	17.800
Lipid in cells (%)	27.8	25.8	47.7
Acetone-insoluble lipid in cells (%)	2.8	3.4	18.2
Liquor analyses			
Ether extract (g)	0.177	- ‡	-
Volatile acids (meq)	1.585	-	-
Nitrogen source [$(NH_4)_2SO_4$] (%)	0.1	0.2	0.3
Length of incubation (hr)	120	72	72

***Physical conditions same as those given in Table IV.**
†Due to losses from volatility it was not possible to determine this value.
‡Not determined.

insoluble fraction of the lipid. The isolation and identification of this fraction has been described by Raymond and Davis and Stewart *et al.* The nocardia produced cetyl myristate (14%) and cetyl palmitate (86%) from hexadecane; octadecyl palmitate (20%) and octadecyl stearate (80%) from octadecane. The identity of the alcohol portion of the wax from octadecane-grown cells was established by saponification and isolation of octadecanol. No waxes were found in the lipid of nocardia grown on glucose, hexane, or tridecane or in the yeast lipid from cells grown on octadecane. The function of these waxes other than as products of cellular storage is unkown, but they presumably pile up as a diversionary process to further oxidation of terminally oxidized intermediates. In the nocardia this pile-up evidently was restricted to C_{16} and C_{18} alkanes because the C_{16} and C_{18} fatty acids are more common to its lipid metabolism.

SUMMARY

Data have been presented which demonstrate the conversion of several n-alkanes into cells of a nocardia and a *Candida* species. The weight yield is very high because of the incorporation of oxygen. Products other than cells were not produced in any significant quantities. A capsular slime polymer was isolated from the nocardia cultures but was not identified.

The composition of the nocardia cells was affected by concentration of nitrogen compounds, oxygen, and the substrate.

As a result of this study it seems reasonable to conclude that the technology necessary for production of cells fron n-alkanes is not too different from that used today for nonhydrocarbon substrates. Further, the utilization of n-alkanes proceeds just as rapidly and with higher yields than are ordinarily realized with nonhydrocarbon substrates. The relatively inexpensive nature of some n-alkanes makes them attractive sources for industrial microbiological processes.

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COMPARISON OF GEOMICROBIOLOGICAL PROSPECTING METHODS USED BY VARIOUS INVESTIGATORS

D.O. Hitzman
Phillips Petroleum Company, Bartlesville, Oklahoma

The microbiological method of prospecting for oil and gas deposits was first proposed by G. A. Mogilevskii in 1937. Since that time interest in this technique has been sustained by rumors and reports of its success as a prospecting tool. The two main postulates involved in the theory of any microbial prospecting technique are: (1) Hydrocarbon gases migrate from a subterranean deposit to the surface; and (2) the presence of these emanations can be detected by the ability of a limited group of microorganisms to subsist on these gases by utilizing the energy of oxidation of the hydrocarbon gases.

While several reviews have been written (Beerstecher, 1954) on the theory of geomicrobiological prospecting, very little has been written on a comparison of the techniques employed. Much of the reported work is available only in the form of patents and general articles giving no details for proprietary reasons. However, enough information is available to discuss the procedures in their broad aspects. For our purposes we will assume the correctness of the theory of geomicrobiological prospecting and we will limit our discussion to the techniques used by various investigators to show the relationship between microbial effects and hydrocarbon deposits. All of these techniques have been published and, in a few cases, limited results of their actual field usage are available. Other techniques or modifications of existing methods have probably been tried but no information is available for discussion.

Climatic and regional effects are very important in survey work but in general are beyond the control of the investigator. However it is necessary to understand their influence on surveying results (Volg and Schwartz, 1958). The following comments present a number of variables which can be controlled by the investigator and describe briefly some of the alternatives available. In all methods an abnormal (high) concentration of microorganisms or an increase in the physiological effects of the organisms determines the apparent location of the hydrocarbon deposit. Thus in the following methods it is implied that increased numbers of microorganisms or products of their reactions show the desired effect.

1. TYPE OF SURVEY

The technique can be designed for reconnaissance-type surveys in which large areas are covered by a minimum number of samples to determine whether any portion of the area has oil potential. Such a survey may cover hundreds or thousands of square miles in a cursory manner. The other alternative is a detailed survey of a limited area in which definite boundaries are sought. Usually many samples are taken to define a potential hydrocarbon-containing formation or the edge of a field, or to determine a possible drilling site. Most investigators plan their method of conducting surveys so that both types of survey can be handled by the same technique by varying the sampling frequency and pattern.

2. MICROBIAL ZONES IN THE EARTH

The depth at which samples can be taken has been divided into three zones by Russian workers (Kartsev *et al.*, 1959). These divisions are arbitrary and will depend on the survey area.

Soil Zone

Up to 10 ft deep where soil organic matter decomposes with a population of aerobic and facultative microorganisms.

Subsoil Zone

From 10 to 300 ft corresponding to a so-called erosion zone—containing anaerobes, aerobes and facultative-type organisms.

Deep-lying Zone

Zone below 300 ft which is poorly aerated and slightly permeable and which contains highly selective microbial forms.

The Russians tend to take deeper samples than American workers. It appears that most investigators favor a depth of 6 ft or less for sampling but want the samples to be from below the grass root level and above ground water. Since the majority of methods favor aerobic organisms, the samples should be in an aerated zone. Thus samples are usually taken at depths which vary according to soil conditions and ease of obtaining the samples with available equipment.

3. TYPE OF MICROORGANISMS SOUGHT

The microorganisms isolated and studied have been divided into 3 groups.

Direct Indicators

These are organisms that oxidize gaseous and vaporized hydrocarbons directly. Examples of these are *Methomonas methanicus, Mycobacterium rubrum* var. *propanicus,* and *Mycobacterium perrugosum* var. *ethanicum.*

Indirect Indicators

These are organisms whose presence in specific environments is suggestive of hydrocarbon deposits but which are not usually thought of as direct hydrocarbon oxidizers. Examples of these are sulfate-reducing bacteria, and hydrogen-oxidizing organisms.

Control Organisms

Organisms whose presence may interfere with or lead to false indications of hydrocarbons because of their ability to produce or to live on combustible gases of contemporary formation. Examples of these are cellulose decomposers and methane producers.

In general the direct indicator organisms are the type most sought by isolation techniques. However, because of the possible recent formation of methane, the use of direct methane indicator organisms is usually avoided if ethane or higher hydrocarbons can be employed.

4. SAMPLING

The method of sampling will depend on the depth, sample size needed, sample location, and number of tests that are to be made. In general, augers are used to obtain the sample or to drill the hole to get side wall cores. However many types of sampling devices have been proposed or employed. Most samples are taken in sterile glass jars but other containers are also used. Proper storage of samples between digging and analysis is a problem. Opinions differ widely on the best way to maintain the sample in an unchanged state. Storage in the cold in a closed container is preferred by most investigators. The length of time a sample can be stored and still give valid results is also open to question. All investigators agree, however, that the faster a sample is analyzed after collection the more accurate will be the results and that all samples collected during a survey period must be analyzed simultaneously.

5. GROWTH MEDIUM

The choice of medium will depend on the type of organisms to be isolated. In most methods a carbon-free mineral medium is used. Although various investigators stress that an excess of certain minerals may make the medium toxic, the mineral media employed in most studies are very similar.

6. SURVEY TECHNIQUES

Investigators have devised the following types of microbial surveys which have been subdivided and named according to the inventor or method of application.

Types of Microbial Survey

1. Soil Surveys
    A. Gas Utilization in Liquid Culture
        1. Mogilevskii Technique
        2. Hassler Technique
        3. Taggart Technique
        4. Sanderson Technique
        5. Strawinski Technique
        6. Maddox Technique
        7. Updegraff Technique
    B. Gas Utilization in Plate Culture
        1. Luchter Technique
        2. Radioassay Technique
        3. Sprinkle Plate Technique
    C. Other Methods
        1. Gas Output Technique
        2. Radioassay CO_2 Technique

   3. Selective Alcohol Technique
   4. Luminescence Technique
   5. Oxidation Reduction Potential Technique
   6. Blau Technique

II. Water Surveys
   1. Russian Technique
   2. Rosenfeld Technique

III. Biologging and Rock Surveys

We will now examine these techniques in more detail in order to show similarities and differences among the various methods. The first use of bacteria as a prospecting tool was undoubtedly proposed by G. A. Mogilevskii in 1937 (Kartsev *et al.*, 1954 and Mogilevskii, 1940). The organisms isolated from soil samples were grown in the presence of hydrocarbon gases and the type, amount, and character of the resulting microbial growth were observed. The procedure consisted in dispersing 5 - 7 g of soil in a mineral medium in beakers which are placed in a bell jar filled with 1/3 methane and 2/3 air or 1/5 propane and 4/5 air. Beakers are prepared for each soil sample. The cultures are examined for growth in 14 days and graded on a 5 point scale as to turbidity and film classification. Other control tests are made for the presence of cellulose-decomposing organisms (Subbota, 1947). This simple test provides the background for all techniques employing gas utilization by microorganisms.

Shortly after the Russians proposed this microbiological theory, G. L. Hassler (1943) described the technique of gas utilization in cultures by microorganisms. His method helped remove some of the arbitrary decisions regarding positive cultures which must have hindered the Russian interpretation of the data. Hassler described a manometric technique in which the organisms in soil samples from above oilfields utilized more hydrocarbon gas (causing a greater change in pressure readings) than organisms in soil samples away from oil deposits. His concept of a manometric measurement provided the basis for most of the later methods although his two patent claims were only for an apparatus to measure pressure in containers.

Immediately after the Hassler application, M. S. Taggart (1948) stated that utilization of hydrocarbon gases by soil microorganisms could be used as a prospecting tool. He measured a change in pressure caused by a growing culture in a short time (2 - 15 hr). The methods of Hassler and Taggart differ very little from each other. R. T. Sanderson (1942) described the method of planting known cultures in soil to observe the effects of gases on them, a method of analyzing soils for hydrocarbophagic and hydrocarbogenetic organisms; and a method of exposing organisms to soil gases for observation of their growth. This patent surprisingly mentions water surveys and products of microbial action as indicator substances. However these microbial techniques are, in general, used only in order to improve the reliability of soil gas survey which Sanderson apparently thought to be the best method.

R. J. Strawinski (1954, 1955) described an improved manometric technique similar to that of Hassler and Taggart. In this method the manometric measuring device is based on the transfer of medium into the reactor to re-establish the pressure equilibrium. This innovation, together with a better understanding of the microbiological growth effects and factors, provides a more workable technique.

Recently (1956), a further refinement of the Strawinski patent has been made by J. Maddox (1959). He suggested that better results can be obtained by a two-stage incubation period. The first stage, an initial incubation under quiescent conditions for 24 - 72 hr which is optimum for cell proliferations, is followed by an agitation stage which is optimum for gas consumption during which the measurable utilization of the hydrocarbon substrate by the hydrocarbon-consuming organisms takes place. Another feature of the modification is the addition of 0.5 to 5 wt. % of a finely-divided surface-active siliceous material such as clay or diatomaceous earth to the slurry of soil and nutrient medium prior to its incubation. Maddox claimed that this additive is used to "eliminate the effect of clay-sand content of the soil sample on the hydrocarbon utilization, which means the rate of hydrocarbon gas uptake is directly proportional to the frequency of hydrocarbon-consuming organisms."

At approximately the same time that Strawinski applied for his patent, Updegraff and Chase filed on a very similar procedure. This patent was withdrawn from issue but in 1958 a patent (Updegraff and Chase, 1958) was issued to these investigators. Their apparatus is a modified manometric technique in which the procedure is designed to eliminate any adaptation of the soil organisms to the utilization of the hydrocarbon gases. They state that adaptation ordinarily occurs more than 25 days after inoculation and therefore all samples which do not show initiation of hydrocarbon consumption within this time should be regarded as negative for hydrocarbon-consuming organisms. The second feature of this patent is a comparison between the time required for the start of consumption of hydrocarbons in soil samples with the time required for known numbers of hydrocarbon consumers to initiate consumption. They suggest use of the organism *Agrobacterium ethanicus* as control.

Since all the early methods (Bokova *et al.*, 1947) are utilization studies which show that the greater the number of organisms the greater the utilization, it follows that attempts would be made to count the number of hydrocarbon-consuming organisms directly rather than measuring their effect. This direct approach was taken by Anna Luchter, who published information (1953, 1958) on the growth of hydrocarbon-consuming organisms on silica-gel plates in the presence of a hydrocarbon gas—air atmosphere. Dilutions of the soil were made and plated in the presence of 1/3 hydrocarbon gas and 2/3 air with control plates incubated in the presence of air only. The difference between the two counts indicates any abnormal concentration of hydrocarbon-consuming organisms. Subcultures can be made of the colonies to confirm their ability to oxidize hydrocarbons.

The plate count method was further refined by J. B. Davis (1957), who used radioactive ethane as the hydrocarbon source for microbial growth. Actually two radioactive methods were employed (Davis *et al.*, 1959). The first measured the utilization of 1% radioethane by soil samples in closed containers. The consumption of radioethane was measured by following the CO_2 production. The CO_2 was isolated after incubation of the soil sample and measured as radioactive $BaCO_3$ with the more radioactive CO_2 indicating increased hydrocarbon oxidation.

The second process was a plate method in which soil dilutions are made on 0.01% soil-extract agar plates which are then incubated in the presence of 1% radioethane in air. The total colony count is made, the agar pads are dried between exposed film to keep them flat, and are placed on x-ray film. The colonies which utilized radioethane and incorporated it into their bodies are identified by the exposure spots on the x-ray film. These radioactive colonies are counted and compared to the total count. Although the possibility exists that some nonhydrocarbon-oxidizing organisms may have utilized radioactive CO_2 released by hydrocarbon-oxidizing microorganisms, this technique still provides a measure of the number and ratio of soil organisms able to utilize ethane.

Another plate count method employed by these investigators (Davis *et al.*, 1959), is termed the "sprinkle plate" technique. In this method 0.1 g of friable soil is spread uniformly on the surface of a 2% washed agar plate while it is rotating. The plates are incubated in the presence of 40% ethane in air for 20 - 30 days. The number of colonies (soil particles) that have typical growth are counted using reflected light. Although interpretation of results by this method may be difficult, it can nevertheless be successfully used as a qualitative test.

In addition to the plate count and gas utilization methods other microbial methods have been employed to determine the presence of indicator organisms. M. S. Taggart proposed a method in 1941 in which known cultures of hydrocarbon-consuming organisms were planted in test bore holes and exposed to the soil gases for 12 - 14 days. Cultures in bore holes which contain no hydrocarbon gases showed little or no growth. A great deal of field work on this technique has been done by Anna Luchter (1958). The method shows a difference between locations off and on known oil deposits, but operational problems are considerable.

A different approach to the measurement of hydrocarbon-consuming microorganisms in soil samples was proposed by D. O. Hitzman in 1959. He used a metabolic product of microbial action on hydrocarbons to isolate the hydrocarbon-oxidizing microorganisms. Dilutions of soil samples are plated in the presence of alcohols having chain lengths corresponding to the chain length of the hydrocarbons which the organisms can metabolize. The alcohol provides a specific carbon source while at selected concentrations it inhibits the growth of organisms that are not able to use the corresponding hydrocarbons.

Methods that use bacterial products have also been used for prospecting. The earliest use of microbial products was proposed by L. W. Blau in 1942 as an explanation for his patent application on a geochemical method. The explanation for this geochemical color change in the soil was attributed to consumption of hydrocarbon gases by microorganisms and to conversion of the gases to high molecular weight compounds. The chemical reagent, such as sodium peroxide, apparently causes a color change by acting on the products of this microbial attack of the gases. In September, 1943, Blau found that hydrocarbon-consuming bacteria or products of their action on hydrocarbons absorb certain wavelengths of light. Soil samples were collected and extracted by a suitable chemical solution such as sodium peroxide. The extract was examined by a spectrograph and the absorptions between 5050 to 5250 A and 5300 to 5500 A were measured. A double hump in these bands seemed to be peculiar to the absorption curve of cultures of hydrocarbon-consuming organisms. However it was not established whether the absorptions at these wavelengths were due to the influence of the bacteria or of their products.

In December, 1943, Blau also discovered that a subsurface oil deposit imparts the property of fluorescing under UV light to the surface soil above. This fluorescence was not due to the oil but to microbial action on diffusing hydrocarbon gases by which the gases were converted into high molecular weight products (probably carboxylic acids). Soil samples were collected, exposed to UV light, and their fluorescence compared with samples having known fluorescence. This provided a measurement of the products of microbial action.

Along these same lines, Taggart in 1948 found that the oxidation-reduction (O/R) potential of the soil was changed over oil deposits. He attributed this to bacterial action on hydrocarbons. The resulting products, assumed to be unsaturated hydrocarbons and nascent hydrogen, imparted increased reducing characteristics to the soil. An oil deposit supposedly can be located by using any O/R measuring device and outlining areas of low O/R potential by this method.

A method proposed by G. A. Slavnina (1948) and M. I. Meisel (Kartsev *et al.*, 1954) suggested the use of luminescence microscopy for identification of a specific type of hydrocarbon-oxidizing organism *(Bacterium aliphaticum liquefaciens)* by its different luminescent effects in UV light and Wood's rays when grown on pentane or higher hydrocarbons. This organism exhibited luminescence on application of special dyes so that the technique could be used to study stages of its development. Other bacteria which oxidize gaseous hydrocarbons do not show this fluorescence.

Other microbial prospecting techniques employ samples other than soil for isolation of the microflora. A technique used by the Russians (Kartsev *et al.*, 1954), with reported success as a reconnaissance tool, involved the analysis of water samples taken below the region of surface contamination from all available aquifers in a region. The water samples can be analyzed for the presence of hydrocarbon-consuming microorganisms by any of the previously mentioned techniques by substituting a water sample for the soil sample. Controls are made to rule out the presence of methane-producing bacteria which could interfere with the analysis. This method had also been briefly suggested by Sanderson in 1942.

Another water analysis technique was given by W. D. Rosenfeld in 1960, in which the water samples were analyzed for the presence of bacteria capable of oxidizing selected liquid hydrocarbons. This method is based on the observation that a correlation exists between nitrate-reducing, hydrocarbon-oxidizing bacteria or sulfate-reducing, hydrocarbon-oxidizing bacteria and oil accumulations. The water samples are inoculated into a mineral medium with a hydrocarbon emulsion for the isolation of the nitrate-reducing bacteria and into a medium capable of growing sulfate-reducing bacteria. A high concentration of these types of hydrocarbon-oxidizing bacteria indicates oil deposits.

K. E. Chave (1959) used the action of sulfate-reducing bacteria indirectly to aid in locating oil. He reasoned that since sulfate-reducing bacteria are commonly associated with hydrocarbons under natural conditions and use sulfate to obtain oxygen for the oxidation of hydrocarbons under natural anaerobic conditions, then the sulfate ion concentration should decrease in relation to other ions in the sample. The concentration of chloride ions, which are least likely to be removed

Table 1. Comparison of Six Geomicrobiological Methods

Method	Mogilevskii	Strawinski	Radioactive Plate	Selective Alcohol	Gas Output	Sprinkle Plate
Essence of method	growth of liquid cultures in presence of gases	manometric measure of gas consumption	measure of incorporation of radioethane	utilization of selective alcohols	exposure of known cultures in core holes	microbial growth on soil particles
Soil sample depth	2–3 m	2+ ft	6 in. to 6 ft	6 in. to 6 ft	3–5 m	6 in. to 6 ft
Soil sample size	250+ g	1 pint	composite sample	100 g	test tubes	composite sample
Soil sample storage	glass jars	42 F tested in 4–7 days	glass jars analyzed within 1 week	glass jars 40 F	–	glass jars analyzed within 1 week
Soil sample handling	–	sieve sample and composite 3 portions	minimum of handling mix with spatula	composite portions	–	minimum of handling mix with spatula
Growth medium	mineral medium	mineral medium	0.01% soil extract in agar plates	mineral medium + alcohol	mineral medium	mineral-salts washed (2%) agar
Growth atm	HC gas—air mixtures	HC gas—air mixtures	1% radioethane in air	air	soil air	HC gas—air mixtures
Technique	5–7 g soil in covered beakers with mineral medium, 5 samples of each soil in bell jar evacuated and filled with gas atm	composite sample blended and 50 cc samples siphoned to gas-filled reactor, the reactor is attached to medium reservoir	soil dilution spread on plate, incubated, colonies counted, the agar pad is removed, dried, and placed on x-ray film, radioactive colonies counted	soil dilutions plated with alcohol mineral medium, incubated and colonies counted	culture of known titer is incubated in core holes and the growth measured after incubation	0.1 g friable soil sprinkled on rotating plate, and the plate incubated, particles with growth observed
Incubation condition	stationary at 32 C for 14 days	quiescent or agitated at 28–32 C for 3–8 days	30 C for 30 days	37 C for 6 days	ground temp. for 14 days	30 C for 20–30 days
Results	growth causes turbidity and film classified on 5 point scale	utilization of gas causes pressure changes which are measured	comparison of total count to radioactive count	colonies counted	growth of cultures signals presence of HC in soil gases	colonies counted using reflected light

from the water, was compared with the concentration of sulfate ions, and the ratio was used to define oil potential.

Still other techniques employing microbial action to locate hydrocarbons are biologging and rock surveys (Beerstecher, 1954). In these techniques the well cores or sections of rock are examined for the presence of bacterial indicators. The cores are treated to remove the outside layer of contaminated rock, then broken open, and the uncontaminated centers are examined for the presence of hydrocarbon-oxidizing or hydrogen bacteria. Their presence indicates a hydrocarbon deposit, a log of the bacterial numbers having been plotted in arbitrary units of intensity of growth. This method appears difficult but is used by the Russian workers.

Obviously some of these methods have not been employed extensively in the field while others have been tried and found to be difficult to operate. Table 1 summarizes the methods on which field results have been published and of which enough is known to apply the technique.

Other geochemical methods suggest that microorganisms play a role in their operability but they are not microbiological techniques and will not be discussed at this time. The development of other microbiological prospecting methods is probable and these should be carefully examined and discussed whenever they are published.

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DETERIORATION OF MATERIALS

Morris R. Rogers, Convenor

Panelists: R. E. Klausmeier
C. Bruce Lee
Milton Manowitz
Louis Beraha
W. Norbert Berard
J. H. McAteer

INTRODUCTION

Morris R. Rogers

Quartermaster Research and Engineering Command, Natick, Massachusetts

It is generally agreed that deterioration consists of an impairment or loss of the functional value of a material or product. Man has been faced with innumerable deteriorative forces since birth. Over the years, he has tried various procedures to circumvent the prodigious losses (both in natural and economic wealth) resulting from the lingering, ravaging, and ubiquitous nature of these forces.

The seriousness of this problem was highlighted during World War II when our Armed Forces lost millions of dollars of essential equipment which became inoperable as a result of environmental deterioration. To the extent that deterioration problems of the war were basically biological in nature, the central importance of certain types of biological information made itself inescapable. It is well known, for example, that a systematic control of biological deterioration rests squarely upon adequate knowledge and understanding of the fundamental life processes of the microorganisms involved. In the absence of adequate information of this type, problems are misconceived and falsely evaluated. Biological and nonbiological effects may also be confused. Imaginary biological problems may be conjured up where none actually exist, while authentic problems of biological origin may be either underestimated or completely unrecognized.

Since World War II and the Korean conflict, scientific studies in different fields of discipline have solved many of the major problems normally associated with microbiological deterioration. Out of the many studies on the deterioration of materials have come, for example, better understandings of the causes of fabric-finish breakdown, the effects of breakdown products in subsequent deterioration of cellulose, and the role of environmental factors in directly breaking down materials. For example these studies have shown that high humidity causes hydrolysis of certain finishes with production of acid which, in turn, causes chemical breakdown of material. Microorganisms, through the elaboration of specific enzymes, oxidize or hydrolyze cellulose, proteins, fatty acids, and polysaccharides in the form of cotton, wool, leather, and plasticizers. Similarly, significant fundamental knowledge has been gained from basic studies including the diversion and dissipation of radiant energy through photochemically receptive materials to minimize actinic changes. Studies on the metabolism of microorganisms and insects have been initiated to determine basic means of minimizing or eliminating biological deterioration.

In a broad sense, it must be concluded that the inroads of deterioration do not exist just in tropical areas during wartime, but in new and unsuspected areas all around us. As we face the problems of deterioration, our objective should be the comparatively simple and highly practical goal of extending the useful life of materials whenever deemed necessary or profitable. The discussions which follow relate to the biodegradability of plastics, cosmetic emulsions, and detergents, and a new process for making cotton resistant to microbiological attack. Other topics are the control of microbiological deterioration in two vastly different areas: missiles and missile components, and fruits and vegetables. It is hoped that the papers presented at this symposium will highlight some of the old problems and identify new problem areas still waiting to be better understood and solved. It is our intent to illustrate and emphasize the ubiquity of deterioration problems, which continue to present themselves as we embark into the age of space where we will be confronted with even more complex, diverse, and challenging problems relating to deterioration.

MICROBIAL DEGRADATION OF PLASTICIZERS

R. E. Klausmeier and W. A. Jones

Quality Evaluation Laboratory, U. S. Naval Ammunition Depot, Crane, Indiana

Prior to 1944 not one publication dealing directly with tropical deterioration of plastic formulations or components of these formulations, could be found. The paucity of early information is not unexpected. Plastics up to that time had enjoyed their greatest use in the temperate zone and had not given any serious trouble. However, during World War II large amounts of equipment, some of which contained plastic materials, were moved into areas more conducive to large-scale tropical attack, and evidence concerning microbial degradation of these materials has accumulated.

Complete plastic formulations contain not only the polymer itself, but also fillers, plasticizer, pigments and stabilizers. Early reports, such as the summary review of Brown (1945), tended to indicate that the resins themselves are essentially funginert. The amount of pigment or stabilizer material in most cases is small and probably of little importance in plastic deterioration due to biological agents. Fillers of textiles, wood flour, and other natural organic material can usually be replaced with fiberglass or other inorganic matter. Plasticizers, however, early indicated as the prime source of nutrient for microbial growth on plastic materials, are essential ingredients for many flexible plastic materials, particularly for polyvinyl and cellulosic formulations. On the average, one pound of plasticizer is compounded with every two pounds of vinyl resin.

Plasticizers are used to effect more or less permanent softening and flexibility of thermoplastic resins. Thermoplastic monomer units are linked lengthwise by strong primary chemical bonds to form polymer chains. The chains are further bonded to each other by weaker secondary Van der Waals forces. The general picture, at the molecular level, is a network of long, tangled and relatively inflexible chains in constant motion.

Plasticizing a resin consists of modifying the secondary forces and thereby relieving some of the rigidity. An effective plasticizer must be sufficiently polar to insert itself between polymer-polymer bonds and form polymer-plasticizer-polymer linkages.

Removal of plasticizer by any means—chemical extraction, physical removal, or biological modification—results in undesirable physical changes, such as increased tensile strength and decreased elasticity (Berk, 1950), or loss in the flexibility (Harvey and Meloro, 1949). In addition, microbial growth, presumably at the expense of plasticizer, can cause changes in the electrical insulation properties of resins (Luce and Mathes, 1951; Leonard and Patouillet, 1951; Teitell *et al.*, 1955).

Although the cellulosics were the first plasticized plastics, it is now estimated that two-thirds of the annual 400 million pounds of plasticizers produced are used by the vinyl industry (Lawn, 1959). A current report (Anonymous, 1959) lists 352 plasticizers made by 75 manufacturers.

Chemically plasticizers are high-boiling organic compounds, usually liquid, and predominantly esters of organic acids or of phosphoric acid. Others are hydrocarbons, halogenated hydrocarbons, epoxides, ethers and various polymers. The nonester type plasticizers probably do not amount to more than 10% of the total plasticizer production, and have received little attention from a microbial susceptibility point of view. This paper is concerned predominantly with the ester type material.

The microbial susceptibility of plasticizers has been determined, under laboratory conditions, almost exclusively with pure cultures or with a mixture of a few species. In some cases, precautions were not used in preparation of inocula, so that some contamination of extraneous "wild" organisms, as opposed to laboratory cultures, were incorporated into the test. Most of the data concerning microbial plasticizer degradation has been based on the ability of the plasticizers to serve as nutrients for fungi, usually on a mineral-salts agar.

The ability of fungi to metabolize esters is widespread. Reese, Cravetz, and Mandels (1955) reported that 95% of 358 cultures examined grew on coconut oil, 90% of 309 cultures metabolized methyl acetyl ricinoleate, and 60% of 82 cultures utilized dihexyl sebacate.

The ability of a large number of plasticizers to support growth, when tested with pure cultures, or a mixture of a few organisms, is relatively good. Brown (1945), summarizing the work of three laboratories, reported that almost half of the 144 plasticizers examined would serve as fungal nutrients. These data include compilations of results using cultures singly (National Bureau of Standards, 1945; Wellman and McCallan, 1945), a mixture of four fungi (Molnar and Leonard, 1945), or a ten-culture composite (Wellman and McCallan, 1945).

Included in the tests were various species and strains of the following genera: *Aspergillus, Penicillium, Rhizopus, Chaetominum, Memnoniella, Monilia,* and *Stachybotrys.*

In general, it was concluded that the following type plasticizers were resistant to fungal attack: esters of short-chain aliphatic dicarboxylic acids (i.e., succinic, adipic); esters of tricarboxylic acids (citric, aconitic, tricarballylic); phthalic and phosphoric acid esters; derivatives of toluenesulfonic acid; glycol and glycolic acid derivatives whose aliphatic acid moieties have carbon chains of less than ten carbon atoms in length; and aromatic hydrocarbons.

On the other hand, the striking susceptibility of fatty acid derivatives was obvious. All derivatives of lauric, oleic, ricinoleic and stearic acids are attacked. In addition, natural oils, such as castor and cottonseed oil, containing glyceryl esters of these acids, will support fungal growth.

In a comprehensive study, Berk, Ebert, and Teitell (1957) determined the ability of 24 fungi representing 13 genera to utilize 127 plasticizers as growth substrates. In general, their results agree with those summarized by Brown: the maleates are fairly fungus-resistant; the alkyl derivatives of phosphoric and phthalic acids do not serve as carbon sources for fungi. Stahl and Pessen (1953) examined a number of plasticizers, utilizing *Aspergillus versicolor* or the bacterium

Pseudomonas aeruginosa in shake flask culture, and using the dry weight of cell crop as an index of utilization. In these experiments it is again noted that citrate, aconitate, phosphate and phthalate esters are funginert.

To date a limited study of plastic films incorporating various plasticizers has verified the data accumulated with the pure plasticizers (Berk, 1950; Harvey and Meloro, 1949; Wellman and McCallan, 1945; Boor and Shurtleff, 1950).

As mentioned, however, almost all of the data has been derived from experiments utilizing laboratory stock cultures. In many cases, the culture was originally isolated from infested plastic material. However, whether or not the isolate was utilizing the plastic or was adventitiously present, growing on contaminating debris, is not known. In some cases, the cultures used were those recommended for use in testing textiles for mildew resistance. In no case was the culture isolated as a result of experimentation designed to isolate microbes capable of active degradation of any particular type plasticizer.

In order to evaluate critically the susceptibility of those materials considered funginert, it seemed advisable to consider two factors: (a) the organisms utilized for testing, and (b) the use of growth as a criterion of microbial susceptibility.

The problem of selection of the proper organisms for testing materials for microbial susceptibility is not new. Berk, Ebert, and Teitell (1957) noted, "Two fungi, *Aspergillus versicolor* and *Aspergillus niger*, commonly used in tests of the fungus resistance of plastic materials, do not produce as much visual growth upon the esters tested as many of the other fungi. From [their] Table 2 it is seen that *Aspergillus versicolor* and *Aspergillus niger* were not able to utilize some of the plasticizers on which other species of fungi, such as *Aspergillus terreus*, *Aspergillus flavus*, *Mucor* sp., and *Trichoderma* sp., showed moderate to heavy growth. Therefore, conclusions concerning fungus resistance, which are based upon studies using one organism, may be misleading."

More recently Nette, Pomortseva, and Kozlova (1959) examined the rubber-degrading capacity of seven fungi utilized in a fungus susceptibility test method adopted by the International Electrochemical Commission in Geneva in 1954. None were capable of degradation of pure rubber hydrocarbons. However, by a type of enrichment-culture method a number of actinomycetes and other bacterial types were isolated which could cause active degradation of pure rubber. One actinomycete actually digested holes in the rubber film directly under the colonies, and, in one experiment, caused a weight loss of more than 50% of the original film in one and one-half months.

The concept of isolation of a particular physiological type of microorganism from a heterogeneous group of microbes by use of the so-called enrichment-culture technique was introduced by Beijerinck and by Winogradsky during the last decade of the past century. By use of the enrichment method we have been able to isolate from soil a number of organisms capable of growth with several of the "funginert" plasticizers. The isolation procedure is that of soil enrichment followed by shake flask culturing through 6 to 10 serial transfers. This method is essentially that utilized for isolation of naphthalene-oxidizing microorganisms (Klausmeier and Strawinski, 1957). To date, enrichments have been carried out using the plasticizers listed in Table 1. In no case was there a failure to isolate cultures. However, as yet the degradative capabilities of most of these

Table 1. Plasticizers Used in Enrichment Cultures

Dimethyl phthalate
Diallyl phthalate
Dioctyl phthalate
Dibutyl phthalate
Octyl decyl phthalate
Tributyl citrate
Dibutyl tartrate
Tricresyl phosphate
Sucrose acetate isobutyrate

has not been reconfirmed. Some of the cultures from the groups carried through the reconfirmation stage (those isolated from dibutyl phthalate [DBP], tributyl citrate [TBC], diallyl phthalate [DAP], and sucrose acetate isobutyrate [SAIB]) did not grow in pure culture utilizing the ester as the sole organic nutrient (Table 2). The most active cultures isolated from DBP (a member of the genus *Fusarium,* labeled 2P3) and from TBC (an *Aspergillus terreus,* labeled TBC-7, and a "black yeast," identified as TBC-2) have been examined most closely with regard to plasticizer degradation.

Table 2. Confirmation of Plasticizer Enrichment Isolates

	Plasticizer			
	DBP*	TBC*	SAIB*	DAP*
Isolates	36	11	20	20
Confirmed	21	5	9	6
Molds	16	1	4	-
Yeasts and Bacteria	5	4	5	-

*DBP, dibutyl phthalate; TBC, tributyl citrate; SAIB, sucrose acetate isobutyrate; DAP, diallyl phthalate.

The DBP-utilizing culture was capable of growth not only with DBP but with a number of other phthalate esters as well (Table 3). The only esters that did not support any growth were diallyl and di-n-octyl phthalate, although growth with dimethyl phthalate was scanty. These data were measured after 7 days' incubation, although in most cases, the results remained constant after 4 days.

Table 3. Utilization of Phthalate Esters by *Fusarium* 2P3

Ester	pH	Growth (mg)
Control	6.7	–
Dimethyl phthalate	6.7	1.6
Diethyl phthalate	4.0	7.1
Dipropyl phthalate	3.8	4.0
Dibutyl phthalate	4.3	3.5
Di-n-octyl phthalate	6.7	0.0
Butyl isodecyl phthalate	5.9	7.3
Diallyl phthalate	6.8	0.3
n-Octyl n-decyl phthalate	6.7	2.0
iso-Octyl iso-decyl phthalate	6.4	4.4

On the basis of preliminary data, it appeared that the ability of this culture to utilize an ester for growth was dependent upon its capacity to utilize the alcohol moiety of that ester (Table 4). Octanol, like dioctyl phthalate, failed to support growth, and both methanol and the dimethyl ester supported only scanty growth.

Table 4. Utilization of Aliphatic Alcohols by *Fusarium* 2P3

Alcohol	pH	Growth (mg)
Control	6.4	–
Methanol	6.4	1.2
Ethanol	6.3	25.7
n-Propanol	4.9	7.3
n-Butanol	6.1	49.2
n-Octanol	6.3	0.0

Using acidogenesis as an indication of growth rate it appeared that DBP was the preferred substrate among the lower molecular weight phthalate esters (Fig. 1). In spite of the ability of this culture to readily utilize the butanol moiety of DBP, not all butyl esters will support the growth of this culture (Table 5).

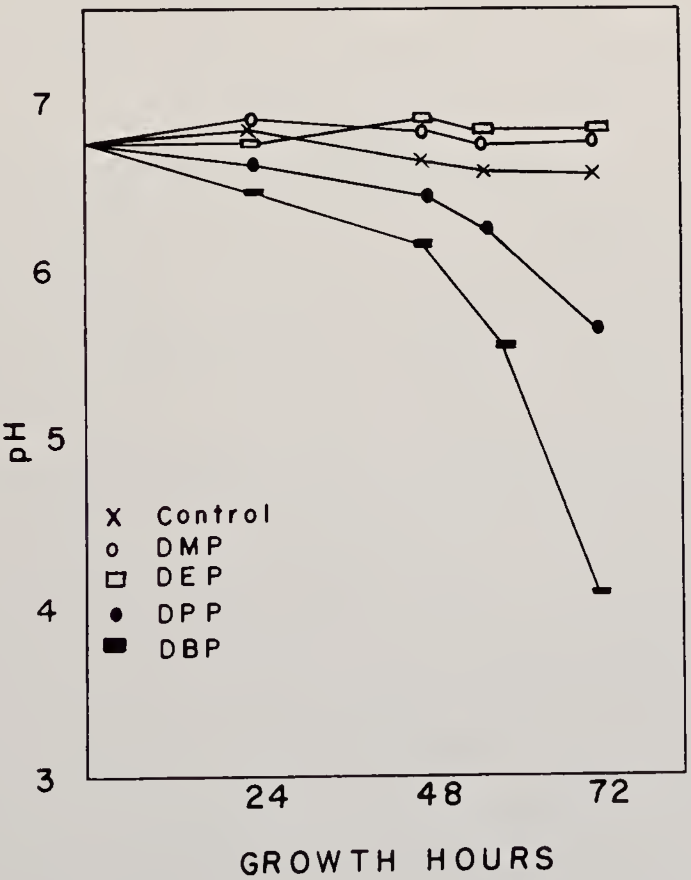

Fig. 1. Acidogenesis from phthalate esters.

Table 5. Utilization of Other Butyl Esters by *Fusarium* 2P3

Ester	pH	Growth (mg)
Control	6.6	-
Dibutyl phthalate	4.2	1.8
Butyl iso-decyl phthalate	6.1	2.6
Butyl oleate	3.6	39.6
Tributyl citrate	6.4	0.0
Dibutyl sebacate	4.0	31.2
Dibutyl tartrate	5.7	0.0

In order to obtain information concerning the mechanism of degradation of phthalate esters, the acid formed during DBP metabolism was extracted from the medium and purified. The acid proved to be an ester of a dicarboxylic acid which was identified, on the basis of chromatography, melting points, and infrared spectrophotometry as the monobutyl ester of phthalic acid. Similarly, the acid formed during dipropyl phthalate degradation has been tentatively identified as monopropyl phthalate. It appeared, therefore, that this organism deesterified one alcohol moiety from the diester, but left the other intact. Why deesterification of the second ester grouping did not occur is not known.

Similar studies with the tributyl citrate isolate TBC-2 indicated that the culture could grow readily with the tributyl and triethyl esters of citric acid, as well as with the acetylated tributyl and triethyl citrates, but could not utilize acetyl tri-2-ethylhexyl citrate. Similarly ethanol and butanol, but not 2-ethylhexanol, supported growth of this culture. This would tend to indicate that, as in the case of DBP degradation, following deesterification, the alcohol moiety of the ester is utilized for growth. In essence, then, it has been shown that at least two of the so-called "funginert" plasticizers will support growth of microorganisms if care is taken to properly select the challenge culture.

There is, at present, no adequate experimental data concerning the advisability of using some criterion other than growth of the test fungus as a measure of the susceptibility of a particular plasticizer. It is well known that microorganisms will grow, in the presence of various types of suitable organic debris, such as dust, spilled foodstuffs, or perspiration, on the surface of materials which will, in themselves, not support growth. It is generally considered that such adventitious growth will not adversely affect the physical properties of the material, excepting such materials as lenses and other optical systems.

However, it is generally accepted that hydrolysis of the plasticizer esters is mediated by an extracellular esterase (Reese *et al.*, 1955; Berk *et al.*, 1957). Can it not be presumed that adventitious contaminants, growing on organic debris, will also liberate these extracellular esterases under proper conditions? Even if these hydrolytic enzymes should be adaptive in nature, it seems likely that they would be formed in the presence of the plasticizer ester present in plastic formulations. It is well known that many compounds which are not the natural substrate of an enzyme will induce its formation in microorganisms. Cohen and Monod (1957) have reported that a series of synthetic β-D-galactosides will induce formation of β-galactosidase. Further, as a group, the esterases are notoriously lacking in specificity. Various substrates utilized in assays of lipases and other esterases include ethyl or benzyl butyrate, acetylsalicylic acid, and p-nitrophenyl acetate. On this basis, it would seem feasible to expect that during adventitious growth of a fungus such as *Fusarium* 2P3 on a vinyl formulation plasticized with dioctyl phthalate, an esterase capable of hydrolysis of alkyl esters of phthalic acid might be liberated. This esterase could then cause degradation of the dioctyl phthalate, even though this phthalate ester will not support growth, growth being dependent upon utilization of the alcohol moiety. This would not preclude growth of other contaminating microbes at the expense of the octanol; in this case total growth on the material would be increased and also one of the products of the hydrolytic reaction would be removed, enhancing complete hydrolysis of the ester.

It is readily conceded that this hypothesis is based upon the presence of debris to support the original growth of the organism. It is based upon conjecture. It is entirely possible that the esterases of the test fungi will be much more specific than the esterases from liver and other animal tissues. However, if these conjectures be only partly correct, it seems that the methods utilized in testing plasticized formulations might need to be revised to incorporate this new knowledge. Indeed, it may be possible to eventually carry out testing with a mixture of a few critical enzymes, thus eliminating growth as a criterion, and decreasing the time for testing to a few hours. Proof either to verify or deny these conjectures, unfortunately, must await further experimentation.

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THE ROLE OF FUNGI IN THE DETERIORATION OF MISSILES AND MISSILE COMPONENTS

C. Bruce Lee

Research and Engineering Directorate, Ordnance-Tank-Automotive-Command, Center Line, Michigan

During the summer of 1957, one of the large missile manufacturers urgently requested information from the Detroit Arsenal Ordnance-Tank-Automotive-Command concerning current available information on the resistance of missiles and missile components to deterioration by fungi. The inquiry was prompted by the discovery that fungus growth had been found on isolated missile materials in temporary storage during manufacture. Although growth was negligible, the presence of fungi was considered a source of potential difficulty in the future tactical operation of missiles. Therefore, a specific request was made for the OTAC to consider a contractual effort to determine the susceptibility of materials, items, and components of missiles and missile systems to deterioration by fungi.

An invitation was extended for us to visit the missile manufacturer in order to define those areas of the missile which might serve as possible nutrition sources for fungi and subsequently to submit suggestions for research investigations aimed at control or elimination of fungus development on missiles and missile components. Microbiological testing of materials was requested by the manufacturer with the objective of eliminating fungi-susceptible items and replacing them with funginert articles so that the completed missile would be a tactical weapon in which any malfunction could not be attributed to the deleterious, degrading action of fungi.

Preliminary inspection at the manufacturing site revealed a large number of heterogeneous materials compounded of items and components. Many were completely foreign to the experience developed over a period of nearly ten years on tanks, tank-automotive vehicles, and their parts, by the OTAC. Materials inspected included the following: natural rubber, synthetic elastomers such as GR-S, butyl rubber, neoprene, buna-N, silicone rubbers, various plastics, plastisols, natural fibers, synthetic textiles, coated and untreated papers, a miscellaneous assemblage of felts, wool linings, packaging materials, gasketings, asbestos pads, coloring agents on electrical tapes, single and multi-conductor cables, sealers, ropes and cordage, pressure sensitive adhesives, potting compounds, greases, tubings, ignition wires, and many others.

There were also assembled and partially completed articles such as control panels, inverters, transformers, distributor panels, cable harnesses, control valves, expulsion units, and relief-valve assemblies. In addition, inspection of disarticulated missiles in various stages of manufacture was permitted, and examinations were made on the articulation and relationships of the various elements to each other. Further, items were inspected that had been returned from storage and operational readiness.

As a result of tours of the manufacturing site, it was possible to define for the manufacturer those materials, items, and components which offered possible sources for fungus nutrition. A complicating factor to the evaluation of the items was that the materials employed in missiles were often of unique engineering design and construction, and it was necessary to consider planning for the special requirements for testing which would be entailed.

Missiles are relatively new weapons in defense, and there are no specifications currently available to determine their resistance to microbiological deterioration. Thus, it was necessary to review and evaluate specifications from all of the federal services for possible adaptations to missiles research. This was not a simple task as there are many available material specifications. A large number are similar in objective, have similar testing procedures, are outdated with no supercession, or are often difficult to employ routinely in the laboratory. It was concluded that many of the items to be tested for resistance to fungi could be tested using several different specifications. Therefore, in preparing the testing proposals for the manufacturer, arbitrary decisions were often made. The proposals relied basically on four major specifications from the Air Force, the Army Ordnance Corps, and the General Services Administration.

In instances where no available specification existed for a unique type of material or component, it was necessary to improvise in the development of a testing method using analogies between previous laboratory investigations on tank-automotive vehicles and the materials supplied from the missile manufacturer.

Concurrent with the decision on specifications, the OTAC laboratory personnel had to consider plans for accomodating the missile items and components to available laboratory facilities. The majority of the missile parts were large, cumbersome, and unusual in shape, and this necessitated a complete change in test procedure in order to handle the equipment in the Detroit OTAC laboratories.

Fungus investigations were made on the Redstone and the Honest John Rockets. The former was manufactured for the Army Ballistics Missile Agency by the Chrysler Corporation Missiles Division, and the latter was a product of the Douglas Aircraft Corporation of Santa Monica, California. Additional research was performed on parts from the Jupiter C rocket and consulting service was done on the Atlas and Nike missiles as a result of requests from the manufacturers and government agencies. The majority of the requests have been concerned with microbiological problems that have developed following manufacture as a result of ready operational storage at various sites in this country and other parts of the world. In addition, information has been solicited on the control of fungus utilization of ground-support missile equipment such as control cabs, trailers, etc.

Almost every phase of the missile program at the Detroit Arsenal OTAC was unusual. For example, where previous fungal investigations on automotive equipment had been conducted with small-sized, hand-portable parts, the research on missiles entailed the use of fork lifts, overhead cranes and parts delivered to OTAC on railroad flat cars and automotive trailers. Further, it was realized early in this project that there was a unique interdependence of components with each other and to the whole assembled missile. Tactical reliability assurance was necessary because once the missile was operative there could be no recall for adjustment or replacement of parts except by absolute destruction of the missile at tremendous loss. This situation can be contrasted to previous activities on tank-automotive vehicles where adjustments and replacements of parts were relatively simple matters.

Prior to actual laboratory investigations, conferences were held with missile engineers, production personnel, and laboratory technicians at the manufacturing site. This procedure had a twofold objective. First, it seemed necessary to acquaint the missile establishment personnel with the importance of fungi as agents of deterioration. Formal and informal presentations included discussions of basic fungal taxonomy and morphology, a review of prior deterioration experiences on tank-automotive items, and an analysis of the specification-testing methods available. Precise information was given on the affinities of certain species of fungi for particular material substrates, the proposed testing times for the missile materials, the testing situations available at Detroit and in other parts of the country. This provided a background for planning the program and obtaining a reasonable allocation of funds.

Secondly, the missile personnel provided blue prints and diagrams of items, discussed the interrelationship of various parts of the missile systems, and sought advice on possible loci of potential deterioration by fungi in the missile and missile components.

Unfortunately, it was not possible to confer with design personnel at the concept — drafting board stage. If this had been done, many of the problems that arose as a result of fungus activity might have been avoided by the microbiologist's supplying information on the use of inert substitutions for materials and suggesting superior designs within the segments of the missile which would preclude future utilization of the parts by fungi.

In order to aid manufacturing personnel in securing additional background in the field of fungal deterioration of materials, lists of basic references and reports on laboratory experimentation from both government and private sources were supplied as guidance aids.

This mutual exchange of ideas, microbiological and engineering, was valuable and resulted in the preparation of nine testing proposals for determining the resistance of missiles to fungi. Each proposal for testing was written to accomodate either a particular class of materials or specific items. The following proposals were written from the Redstone Missile:

1. Fungus tests conducted on the 1800 VA inverter.
2. Tropical environment tests of plastic materials.
3. Fungus tests of top measuring distributor, tail measuring distributor, launcher relay box.
4. Fungus tests of multiconductor cables.
5. Microbiological test on elastomers compounded at the Detroit Arsenal OTAC.
6. Fungus test of miscellaneous materials at the Detroit Arsenal OTAC.
7. Tropical environment tests on standard elastomer samples received from vendors.
8. Fungus test of an assembly of diverse missile system components.

The following proposal was written in conjunction with the microbiological testing of the Honest John Rocket:

1. Environmental test specification for weatherized Honest John, Type II, Rocket.

Wherever possible, information was sought from the manufacturer on the composition of supplied materials. However, considering the large complexity of items supplied to our laboratory, this was not often possible. Thus many components were placed into testing with scant information as to composition. Items were frequently delivered with such simple designations as "plastic," "elastomer," "gasketing." An additional complicating factor was a lack of information concerning quality control of items during manufacture. Data was not available on the use of additives, alterations in surface character as a result of the use of heat, chemicals and other finishing agents, and the application of coatings and protective materials for storage; many of the preceding being capable of supporting fungus growth and thus rendering possible false interpretations on the actual resistance of materials to utilization by fungi.

Therefore, it was necessary to consider the following points in writing testing proposals:

1. Determination of the composition of materials submitted to test, either by verified "tech" sheets from the supplier or by laboratory analysis.
2. Precise estimation of fungus utilization of test materials; decision whether growth was adventitious or actually degrading.
3. Recommendations for particular performance tests on materials to define actual microbiological utilization of items and a concurrent correlation of this information with visual observation of fungus growth.
4. Specific determinations of species of fungi indicated as degrading missiles materials.
5. Suggestions and methods to be devised to demonstrate the effectiveness of protection of materials against fungi and possible necessary corrective measures.

The majority of the materials available for microbiological testing were submitted to OTAC through regular supply channels from the manufacturers. However, in isolated instances, the manufacturer requested OTAC's cooperation in the development of items for specific missile applications, and the resulting products were tested for their resistance to fungi.

At the inception of the actual investigations on missiles, decision was necessary as to the testing site. It was suggested that the missiles be shipped to actual tropical rain forests such as occur in Panama. However, because of internal policies over which there was no control, the actual tropical environment situation was abandoned, and it was decided to use the simulated tropical testing facility at the Detroit Arsenal OTAC. This is a chamber approximately 15 x 20 x 9 ft, with an access door 8 x 8 ft. Using this room it was possible to accomodate the missiles and components submitted for test. In the case of microbiological exposure of a completed missile such as the Honest John, it was necessary to disarticulate the rocket to fit it into the chamber.

The tropical chamber was instrumented to simulate the conditions of the tropical rain forest. There were 8 hr of diurnal conditions and humidities, 8 hr of nocturnal conditions and humidities, and two 4-hr periods of transition between the day and night conditions. The specific temperatures and humidities are as follows:

1. Nocturnal: 70 ± 2 F, R.H. 92% to saturation.
2. Diurnal: 86 ± 2 F, R.H. 92%.

An automatic cam was attached to the instrumentation of the room and the nocturnal, diurnal, and transition conditions duplicated on a 24-hr cycle.

The fungus population within the room was assured using soil beds of rotting humus. In addition, the walls were banked with soil containing decaying leaf mold composted at regular intervals with well-rotted equine and bovine droppings.

Because of the specificity of certain important species of deterioration fungi used on military materials for particular substrates, non-fungicide-treated cardboard, canvas, rope, cordage and paper were placed periodically in the tropical room. In addition, the media and some expendable items from completed tests on automotive vehicles were scrapped routinely onto and into the banked soil. The two lengths of the room were lined with roll-away oak shelves with wooden slats to allow for complete circulation of the atmosphere, and fungal activity and presence in the room were checked bimonthly using Petri dishes containing various media.

During manufacture of the missiles it was noted that workers installed components into the missiles directly from stocked supplies and there was never any attempt to clean or alter surfaces in any manner. The component was assumed ready for use once it had arrived on the line. This fact was used as a guideline in conducting the research at OTAC. Although it was realized that many specifications in the past called for prior treatment of materials using water baths with controlled pH and temperatures, and chemical cleaning, these steps were not employed with missiles materials. It was believed that any preincubation cleaning would create barriers to securing accurate estimations of resistance of materials to utilization by fungi. Therefore, in all of the testing performed there was no pretreatment of components and all were placed into the simulated tropical environment in the "as-received" condition. In rare cases when the surface of an item revealed obvious adventitious adherents, adjudication for use rested with the manufacturer and such information was added to the logbook.

In the approach to testing, it was necessary to decide on choice of fungi and the means by which fungi were to be employed. Two approaches were possible. The first is that used in various government laboratories: materials received for fungus resistance testing are not inoculated with spore suspensions, but simply placed into the incubation chamber to take advantage of the fungal population of the atmosphere.

However, at OTAC a second technique was employed, and it was decided that all materials for test would receive a complete inoculation with a mixed spore suspension of six to ten species of fungi of known degrading ability on particular types of materials. With this technique, it was hoped that there would be a double security for demonstrating material utilization by fungi; first, from the fungi present in the atmosphere, and secondly, from the wide-spectrum spore sus-

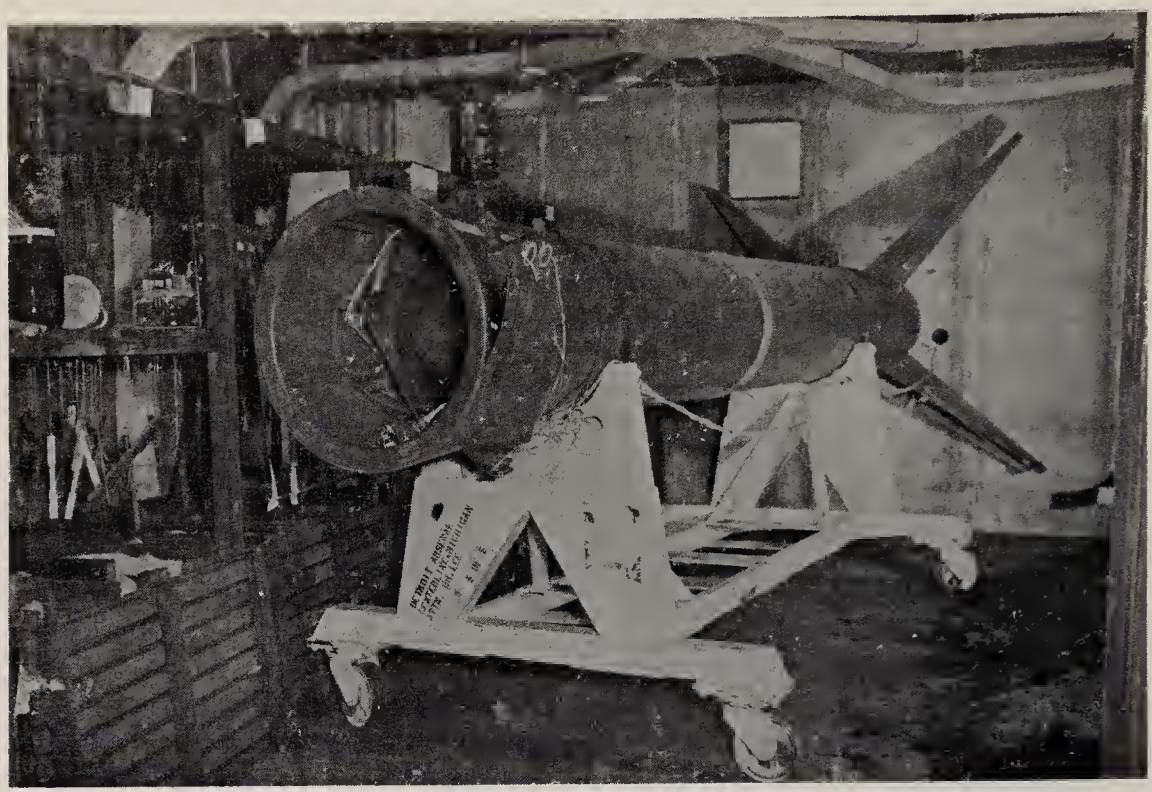

Fig. 1. Honest John Rocket motor section positioned for microbiological exposure in Tropical Chamber of Detroit Arsenal, Center Line, Michigan.

pension sprayed directly onto the surfaces of the materials to be tested. Since in many instances the exact composition of the materials under test was unknown, it was felt that a wide-spectrum spore suspension would be a more effective inoculating agent than the spores borne in the atmosphere of the room. The spore suspension used in the missiles research contained species of the deuteromycetes and ascomycetes that had a proven capability to degrade materials whose major constituents were protein, fats, carbohydrates, etc. The great size of the missiles and the missile components necessitated the preparation of spore suspensions in gallon lots. For example, the Honest John required 5 gal of suspension in order to inoculate completely the missile.

Two methods were used in estimating fungus utilization of materials. The first was visual observation of fungi actually growing on the item under test. This visual observation was complemented by performance tests, which were given to all materials at the inception and at the conclusion of testing. Basically, the performance test consisted of an estimation of the physical, chemical, mechanical, and electrical properties of the materials. Physical, mechanical testing measured tensile strength, abrasion resistance, adhesion, etc. Electrical properties were determined using high potential tests and other data secured from instrumentation. Chemical testing was employed where it was germane to define the basic composition of items and to indicate the presence or absence of surface-applied finishes and extraneous matter. Thus, the testing program usually provided pre-test acceptance limits and information on changes.

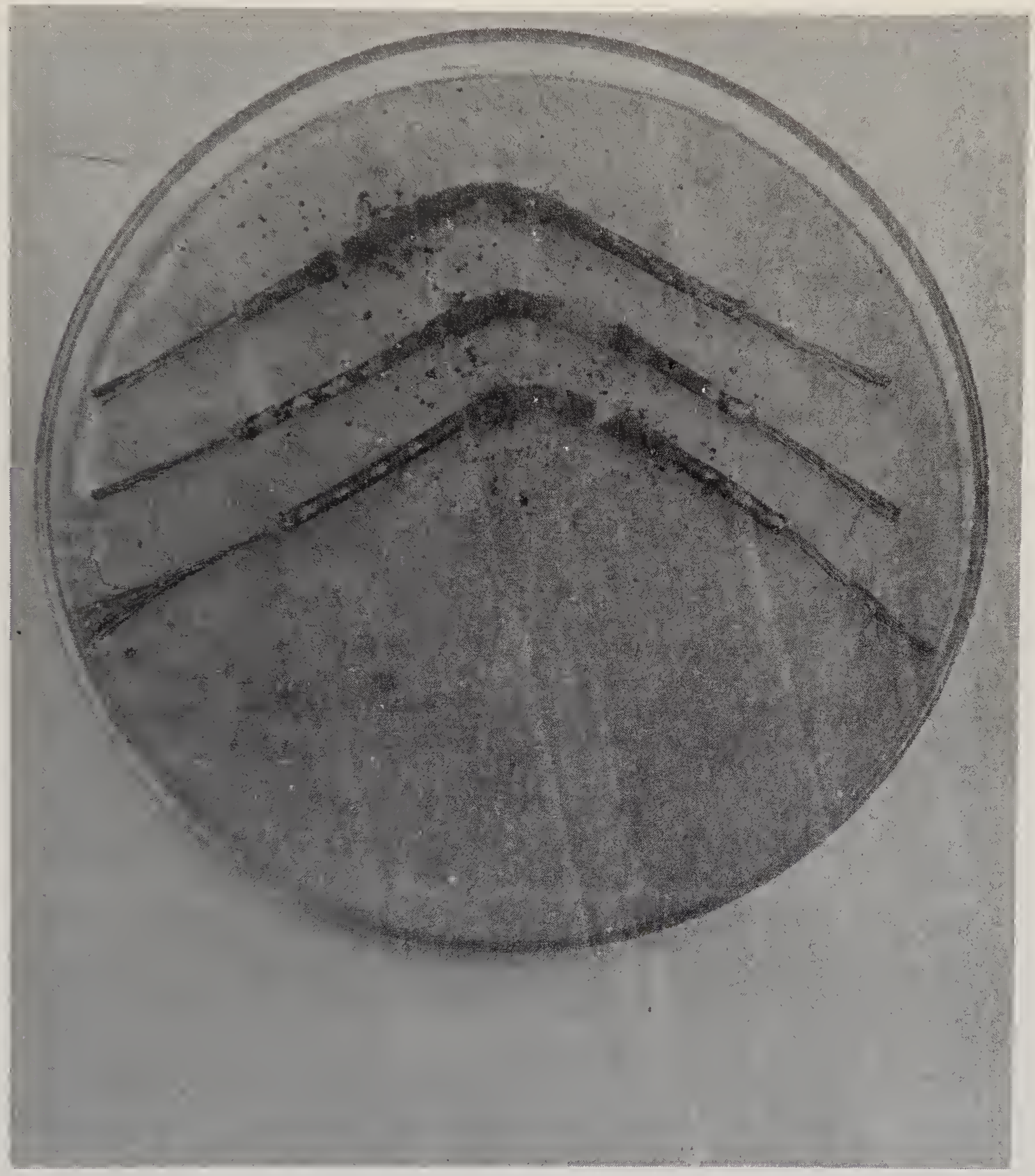

Fig. 2. Cable leads following 30-day incubation with mixed spore suspension of fungi.

Years ago many fungicide test specifications prescribed a 7-day incubation period following inoculation with fungi. Subsequent specifications called for 14 and rarely 21 days. Current incubation times specify 28 days and one ordnance specification indicates an incubation period of 90 days. This longer period was used in the Detroit project except when shorter periods were desired by the manufacturer. The 90-day period was employed as it was believed that shorter testing periods might not be satisfactory for indicating true resistance of missiles materials to microbiological deterioration. Further, since the missile manufacturer requested maximum assurance against fungus activity on com-

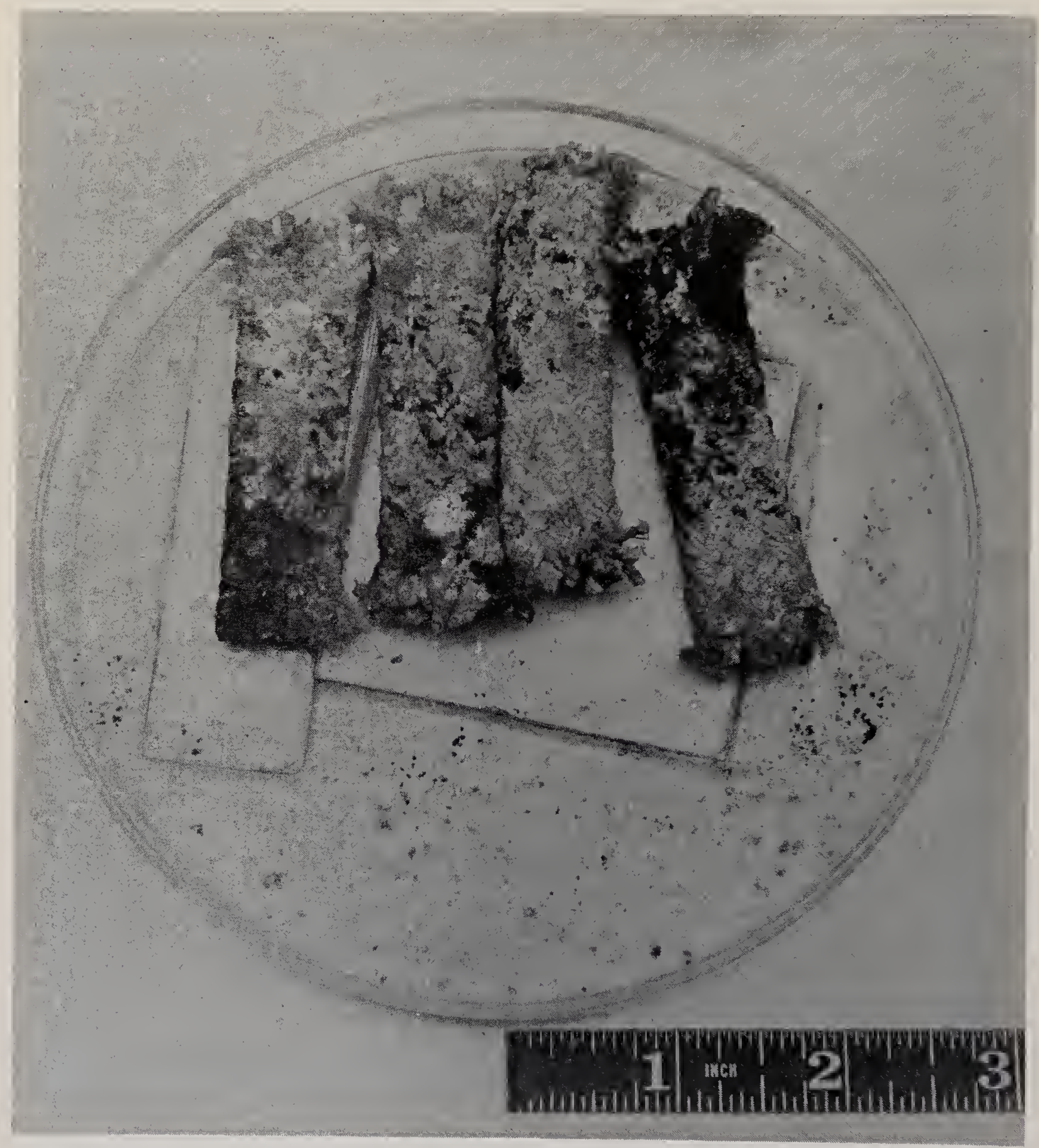

Fig. 3. Asbestos tubing adulterated with cotton following 90-day incubation with mixed spore suspension of fungi.

ponents, the longer period was viewed as one means of compliance with this request. In addition to the pre-test rating for all components wherever possible, each item was supplied in sufficient quantity so that a single specimen might be withdrawn from a group under test and tested at the conclusion of the first 30 days of incubation. Another specimen would be withdrawn for performance rating at the conclusion of 60 days, and a final performance test would be secured at the end of 90 days.

The results of the testing of several of the materials by the written proposals supported the use of the longer testing time. In the elastomers, cable harnesses,

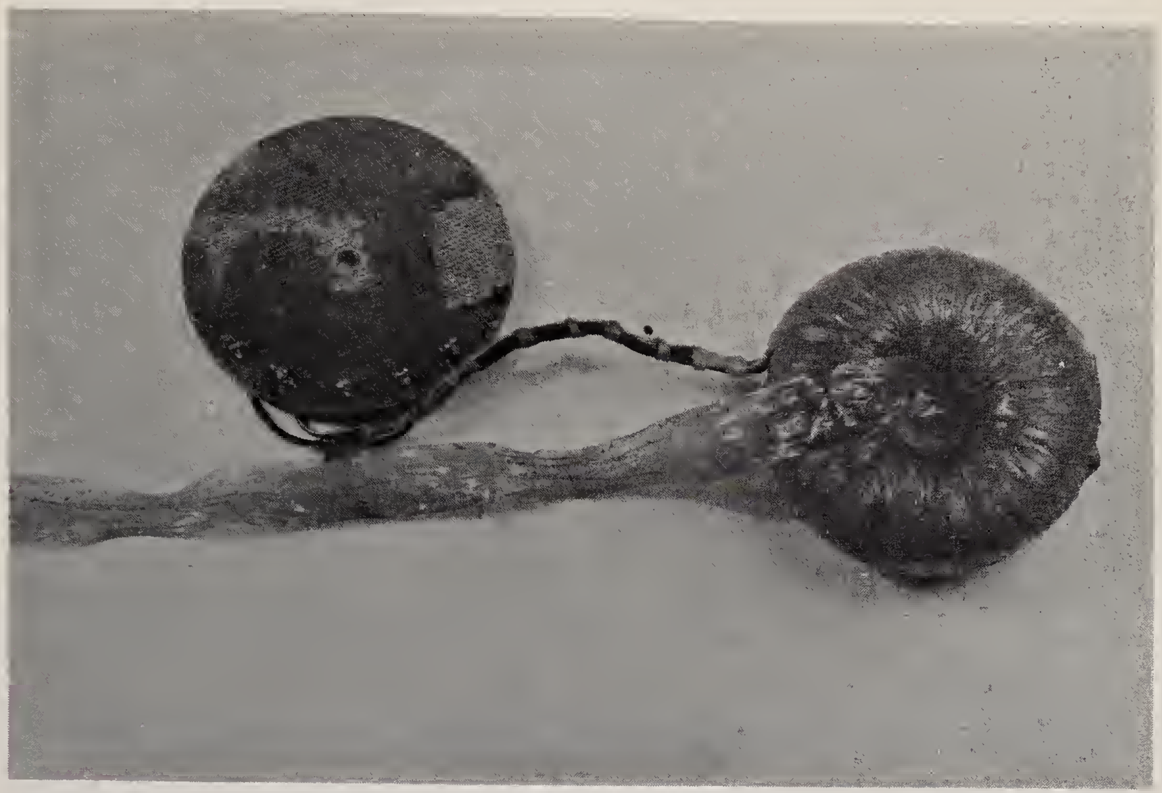

Fig. 4. Transformer following 90-day exposure to microbiological attack in the Tropical Chamber of the Detroit Arsenal (1800 VA Inverter component).

and assembled and disassembled components, termination of the incubation at 30 days would have given a false impression of actual resistance to fungus attack. All of the preceding materials were allowed to continue in testing for either 60 or 90 days and only with the prolonged time did they demonstrate susceptibility to fungi by visual observation and performance test.

Further, many of the materials supplied for test were given a terminal manufacturing treatment with storage protection coatings and finishes. Such items required the longer test time to adjust to the conditions of temperature and humidity in the tropical chamber, and usually there was fungus development only after the 60-day period. The use of the longer testing time at the Detroit Arsenal OTAC laboratory probably accounts for seeming inconsistencies between the OTAC results on materials and results secured elsewhere using shorter testing times.

Also, the longer testing time was interpreted as advantageous for securing simulated storage data. Various project engineers requested expected service-storage life data. The 90-day test data was used in answer to this type of request.

Placement of specimens in the tropical chamber was important. Small items were placed onto the slatted shelves. Larger components were supported by wires. Whole missiles were rolled into the test chamber on a dolly.

Aside from the performance testing of all materials wherever possible, the visual examination of post-test specimens was carefully made. Growth was reported on any surface area and included descriptions of the type of growth, its intensity, and any unusual appearance such as changes in color and colony character. Specimens supplied with cut edges and discreet interstices were examined carefully for any fungi present. All discoloration was checked for fungus origin and unsealed components were opened and articulated to reveal any microbiological development internally. In addition, sealed components were also opened to check for fungi and to reveal if there had been any development of fungi through deterioration of the sealing devices. Materials used for padding on missile surfaces were removed for examination of growth, and interpretation was required where there was evidence of obvious growth, such as fingerprints that had developed fungi, etc.

Exceptional growth was noted for the following items: elastomer buttons compounded by vendors and at the Detroit Arsenal OTAC labs, cable leads containing chromatic materials in insulations, asbestos tubing apparently adulterated with fungi-susceptible cotton, code-colored cable harnesses, silk-wrapped transformers which failed to use potting compound to seal out the simulated tropical atmosphere of the tropical chamber, and solenoids wrapped in natural textile. In addition many of the assembled control panels and other instrumentation developed fungi at various loci.

PRESERVATION OF COSMETIC EMULSIONS

Milton Manowitz
Research Laboratories, Givaudan Corporation, Clifton, New Jersey

The problems encountered in the preservation of cosmetic emulsions have continually increased with the tremendous expansion in the development and use of cosmetic preparations during the past decade. The various organic components and the presence of large quantities of water in these emulsions provide an excellent environment for the growth of microorganisms and most products in use today require the protection of an effective preservative.

Emulsions of the water-in-oil type are relatively resistant to attack since the continuous oil phase acts as a barrier to the penetration of microorganisms into the water phase and also impedes the spread of growth through the system (Tice and Barr, 1958). Oil-in-water emulsions, the most commonly used systems, however, can present an ideal substrate for microorganisms depending on the nutritive value of the various lipid, carbohydrate, and protein derivatives, surfactants and other organic materials which they contain. The visible destructive effects of microbial attack on cosmetic emulsions can take many forms, the most common being changes in viscosity, discoloration, formation of gas and malodor, or complete breaking of the emulsion. The presence of an unsightly mass of fungus growth on the surface of a cosmetic preparation disclaims the elegance of such a product. In many instances, an emulsion might show no visible evidence of microbial attack yet contain an actively growing bacterial population. Introduction of such a material into intimate contact with the skin gives rise to the possibility of infection, and may be classified legally as adulteration under the Federal Food, Drug and Cosmetic Act. The microbial degradation of cosmetic preparations can result in large direct losses to manufacturers who must withdraw such products from the market and replace them with uncontaminated effectively preserved products. Indirect losses also result from future consumer resistance to products with which they previously have encountered spoilage problems and from damage to the manufacturers' reputations.

There has been no systematic identification of the microorganisms found growing in cosmetic formulations, but the following have been prominently listed among those considered responsible for attacking these emulsions (Sagarin, 1957): *Penicillium, Aspergillus, Rhizopus nigricans, Mucor mucedo, Botrytis cinerea, Alternaria, Stemphylium, Cladosporium, Bacillus subtilis, Bacillus mycoides, Aerobacter aerogenes, Pseudomonas* species, and coliform bacteria. Undoubtedly the list could be increased many fold by microorganisms which can and do grow on these products, but spoilage problems are handled with a minimum of publicity by manufacturers who often develop satisfactory solutions without proper identification of the attacking organisms. Microbial contamination of the emulsions during manufacture and packaging occurs through infected plant equipment, water, raw materials, air, and containers. The water-softening devices used in cosmetic plants have been reported to provide a reservoir of actively growing bacteria which can be continuously introduced into the water (Baker, 1959). Since a cosmetic emulsion is usually a complete medium for the growth of microorganisms it must be prepared and packaged completely free of all microflora, or the addition of an effective preservative is required. The presence of only a few unobserved viable cells in the finished item can cause its complete destruction during storage in a warehouse or on a store shelf. The most stringent controls and aseptic conditions employed in the industry often have failed to produce absolutely sterile products, and the incorporation of a preservative has become a necessary adjunct to sanitary manufacturing practices. The addition of a preservative also provides protection under use conditions when the consumer is constantly introducing various microorganisms into the unused portion of the product.

Selection of a preservative for use in a cosmetic formulation requires consideration of various properties in addition to antimicrobial effectiveness (Gershenfeld and Pearlstein, 1939). The compound must be nontoxic, nonirritating, nonsensitizing and compatible with all ingredients in the formulation. It should be colorless, odorless, and stable as well as economically and easily incorporated into the emulsion. These requirements are analogous to those of industrial preservatives for many different applications, except that the irritation and sensitization properties must be investigated more extensively. A valid evaluation of the effectiveness of a compound for use as an emulsion preservative can be accomplished only by testing in the finished cosmetic preparation under consideration. Existing screening tests in agar or broth which determine a compound's inherent antimicrobial activity are helpful only in eliminating completely inactive materials. They will not predict with any reliability, performance in a cosmetic emulsion. Compounds with relatively low activity often prove to be more effective as cosmetic preservatives than materials which have demonstrated extremely high antimicrobial properties in screening tests. The discrepancy between activity in aqueous media in screening tests and performance in emulsion systems can be explained to a large extent by the oil-water partition properties of the compounds. Protection of an oil-in-water emulsion requires that lethal levels of an antimicrobial agent be present in the aqueous portion of the system to contact the invading microorganisms. Antimicrobial compounds with high oil-water partition coefficients often concentrate in the oil, with insufficient quantities being distributed to the aqueous phase to inhibit microbial growth. These compounds cannot protect an emulsion despite their strong inherent antimicrobial properties. Materials with very low oil-water partition coefficients can function as emulsion preservatives at concentrations approximating those levels found effective in screening tests. Thus, a compound whose minimum inhibiting concentration in screening tests is 1000 ppm may protect an emulsion at a level of 0.1 to 0.2% while another agent which inhibits growth at 20 ppm in screening tests can fail to protect an emulsion even at a concentration of 0.4%.

The test organisms used to evaluate emulsion preservatives vary with the laboratory conducting the study and the specific products under test (Rdzok *et al.*, 1955; deNavarre and Bailey, 1956; Charles and Carter, 1959). Often it is feasible to employ the organisms which continuously have been found attacking a particular formulation or to use an aliquot of a spoiled emulsion as the inoculum. Although it is beneficial to use as large a spectrum of organisms as possible, reliable results usually can be obtained with a few representative species if they are carefully selected. All evaluations should include the two ubiquitous organisms *Aspergillus niger* and *Pseudomonas aeruginosa* which are known to attack a large variety of cosmetic and pharmaceutical products over a wide pH range and have comparatively high resistance to antimicrobial agents. Compounds that are capable of inhibiting the growth of these organisms in a cosmetic emulsion usually can be expected to function as effective preservatives.

Incubation of the inoculated formulations in these tests are carried out at 30 C to 35 C for extensive periods up to the anticipated storage life of the product. A minimum incubation period of four weeks should be utilized to determine the possible loss of antimicrobial potency with time. Reinoculation of the samples during incubation often uncovers limitations with preservatives which are being tested at borderline concentrations. Visual examination of the test formulations normally shows the presence of fungus growth, but plating-out techniques in agar or broth are required to detect bacterial growth in the absence of visible emulsion deterioration.

A variety of different types of compounds have been used or recommended for the preservation of cosmetic formulations. Many powerful antimicrobial agents, such as pentachlorophenol and the organic mercurials cannot be used in this application due to limitations in physical properties or toxicity hazards. Benzoic acid (Reddish, 1957) and salicylic acid were employed as preservatives in former years but their relatively weak activity has led to replacement by more potent compounds. Various chlorinated phenols have been used successfully for these purposes with p-chloro-m-xylenol (PCMX) receiving extensive acceptance in Europe as a cosmetic preservative (Joseph, 1952). Dichloro-m-xylenol, chlorinated thymols and carvacrols, o-phenylphenol (Schimmel and Husa, 1956), dehydroacetic acid (Bandelin, 1958), and esters of vanillic acid (Pearl, 1950) also have been suggested or used for the protection of these emulsions. Quaternary ammonium compounds have demonstrated satisfactory preservative properties in many cosmetic products, but careful examination must be made of their compatibility with the components of any formulation, particularly anionic molecules, before they can be safely incorporated. Sorbic acid (Puls *et al.*, 1955) has been highly recommended as a cosmetic preservative in recent years, following acceptance of its use in preventing mold growth in food. However, the antimicrobial properties of this compound as with most organic acids depends on its existence as an undissociated molecule and its activity is significantly reduced in systems as the pH rises (de Navarre, 1959). Currently the most widely used compounds for the preservation of cosmetic products are the alkyl esters of p-hydroxybenzoic acid. These compounds originally were introduced in 1924 in Europe by Sabalitschka (1924), who published extensively on their antifungal and antibacterial activity. The methyl, ethyl, propyl, and butyl esters are the derivatives most commonly used, although studies have demonstrated that antimicrobial activity rises with increased chain length through the heptyl ester (Huppert, 1957). A comprehensive review of the multiple applications of these compounds in the cosmetic, pharmaceutical, and other industries was published by Neidig (1944). They have a wide spectrum of activity against fungi, Gram-positive and Gram-negative bacteria, and show little loss in effectiveness over a pH range of 4 to 8 (Aalto *et al.*, 1953). They are stable, relatively colorless and odorless, have a low order of toxicity and at concentrations up to 5% show no evidence of primary irritation or sensitization on repeated applications (Matthews *et al.*, 1956). Mixtures of the esters of p-hydroxybenzoic acid often are used as preservatives on a pragmatic basis, and there are studies showing the superior properties of such mixtures over the individual compounds (Schimmel and Husa, 1956; Littlejohn and Husa, 1955). In general, the mixtures are active against a greater number of organisms, their activity is additive and sometimes potentiation can be demonstrated (Boehm, 1959).

During recent years, the many advantageous properties of nonionic surfactants, such as versatility, application over a wide pH range, low toxicity, and low irritation has led to their widespread use in cosmetic products. This application of nonionics has been accompanied by increased problems in emulsion preservation, and investigations in the field have concentrated on studying the effects of these surface-active agents on preservatives. It has been reported that nonionics interfere with the antifungal activity of several preservatives (Bolle and Miramanoff, 1950). Lawrence (1953) and Erlandson (1953) found that nonionic wetting agents inactivated the bacteriostatic action of bisphenols and that Tween 80 was a more effective antidote than serum for nullifying the antibacterial properties of these compounds. Extensive investigations with 40 nonionics and 20 preservatives led to the conclusion that practically all nonionics based on ethylene or propylene oxide condensates with each other, fatty esters, alcohols, or acids, inactivate all presently usable drug and cosmetic preservatives (deNavarre and Bailey, 1956; deNavarre, 1957a, 1957b). In a study with 36 nonionics and 26 preservatives, Wedderburn (1958) found that the nonionic surfactants reduced the efficiency of all preservatives when the ratio of surfactant to preservative exceeded certain critical values.

The capacity of the nonionic surfactants to impair the antimicrobial properties of phenolic and most other commonly used preservatives appears to be a well confirmed phenomenon but the mechanism and the conditions under which this inactivation takes place still require further elucidation. Higuchi (1954) and co-workers (Guttman, 1956) demonstrated the incompatibility of phenolic preservatives with polyethylene glycols which could be attributed to the association of acidic hydrogen groups in the preservative with electrophilic oxygen in the glycols. Based on these and other studies, several investigators (Barr and Tice, 1957b; Beckett and Robinson, 1958), have suggested that inactivation is attributable to molecular complexing of the preservative by the polyoxyethylene group present in most nonionic surface-active agents. Another popular explanation for inactivation is based on the solubilization of the preservative within the micelles of the surfactant. Dyer (1958) in a paper presented at the 1958 meetings of the Society for Industrial Microbiology considered the surfactant micelles as a separate phase in the system and suggested that preservatives distribute themselves between the micelles and the aqueous phase according to their partition coefficient. Thus, preservatives having high solubility in the micelles can be inactivated in their presence due to insufficient quantities being available in the aqueous phase to inhibit microbial growth. There is evidence supporting both complex formation and micellar solubilization as mechanisms of the strong inactivation of phenolic compounds and perhaps both should be considered as playing significant roles in this phenomena. As suggested by Kostenbauder (1960), a micelle of nonionic surfactant might provide an ideal model for complexing with a phenolic preservative providing both the possibility of hydrogen bonding and a nonpolar moiety for interaction. Preservatives which would not appear to complex with nonionics might be inactivated solely by micellar solubilization and thus be less easily inactivated than phenolics.

Studies of nonionic inactivation of preservatives for the most part have been concerned with the qualitative aspects of the problem. The presence or absence of growth under varying conditions in synthetic laboratory media has been recorded. Kostenbauder (1960) and associates (Patel and Kostenbauder, 1958; Pisano and Kostenbauder, 1959), investigated the quantitative aspects of the binding of p-hydroxybenzoic acid esters by nonionic surfactants which they related to the concentration of unbound p-hydroxybenzoate necessary to inhibit microbial growth. Using a nylon dialysis membrane, they determined the quantity of free preservative present in aqueous systems containing varying amounts of nonionics as a means of predicting the concentration of preservatives which must be added to a nonionic system to be effective. Nonionic inactivation data was extended from laboratory media to practice by Charles and Carter (1959) who determined the antimicrobial activity of p-hydroxybenzoates and sorbic acid in several typical cosmetic emulsions containing known quantities of nonionic surfactants. Results of this work indicated that the preservatives were more active in the finished emulsion than would be anticipated from data obtained from testing the preservative and nonionic in a synthetic laboratory medium. Their investigation again showed that to obtain a valid measure of the protection provided by a preservative in a cosmetic emulsion it is important to test the compound in the finished product with all components present. A further complication arising from the use of nonionic surfactants was demonstrated by Barr and Tice (1957b), who showed that nonionics of the fatty acid type can be decomposed by microbial growth. Organisms such as *Aspergillus niger* and *Pseudomonas aeruginosa* were shown to produce esterases capable of hydrolyzing the surfactant with the liberation of free fatty acid, thus destroying emulsification properties.

There obviously are many factors present in cosmetic emulsions which can nullify the antimicrobial properties of compounds incorporated into these products. Water solubility, oil-water partition coefficient, and nonionic inactivation all have significant influences in determining the ability of a compound to protect such systems. These factors, although considered separately, are closely related and might be considered as only different measures of a general characteristic of a compound. Some evidence already has been accumulated showing correlation between water solubility and nonionic inactivation; the lower the solubility the greater the inactivation (Dyer, 1958). Oil-water partitioning and water solubility are closely connected and thereby the three factors are linked although undoubtedly this is a superficial version of the relationship. However, these properties bear strongly on the performance of a preservative in an emulsion system since they control its concentration in the aqueous phase and thus its availability to inhibit microbial growth. Perhaps efforts toward the development of more effective emulsion preservatives in the future should be directed at water-soluble compounds.

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CONTROL OF DECAY OF FRUITS AND VEGETABLES DURING MARKETING

Louis Beraha, M. A. Smith, and W. R. Wright

Market Quality Research Division, AMS, USDA, Chicago, Illinois

INTRODUCTION

In order to appreciate fully the problem of decay of fruits and vegetables, it should be remembered that 250 diseases have been found in these perishable commodities during transit or shortly after their arrival at destination.

The cost of these diseases affects everyone. If a conservative estimate of 3% is made of microbial spoilage at the time of unloading at destination, and is applied to the more than 500,000 rail cars of fresh fruits and vegetables shipped each year, the vast losses to consumers, shippers, and producers can be more nearly understood. Figures are not available for ship, air, or truck transport. Actually, as much as 20% of all fruits and vegetables grown and carried through normal wholesale and retail channels are never consumed because of spoilage due to decay. Losses in marketing mean that the broker or agent gets a lower price for the commodity on its arrival. Widespread losses due to decay might result in a shortage of the commodity with an ensuing higher price per unit at the retail level.

Decay of fruits and vegetables in marketing is often the end result of the interplay of various preharvest and postharvest factors. In many instances the actual entrance of fungus or bacterial parasites occurs in the field early in the development of the fruit or vegetable.

The inoculum is in the form of fungus spores, vegetative mycelium, or bacterial cells which infest the soil and decaying plant debris. It may be spread by wind, splashing rain, farm implements, insects, nematodes, or man himself. At certain temperature and moisture conditions, penetration may take place directly through the cuticle, through natural openings such as stomata or lenticels, through flower blossoms, or through mechanical injuries.

While conditions favorable for infection may be present in the field, decay may not actually begin until after the produce is harvested and shipped. There are several reasons for this. First, temperatures may be such that the symptoms are masked or the latent period of infection (incubation of the organism) is extended. Second, the relationship of host and pathogen may not be mutually satisfactory and the expression of disease awaits maturity or chemical changes of the produce to take place after harvest. Finally, the invading organism may not be able to penetrate the unbroken skin of the fruit or vegetable. Careless handling, such as may occur in picking or grading, or from broken packing containers, or chemically induced injuries may provide the avenues of entrance for decay pathogens. Injuries can also result from rough handling in trucks, rail cars, or airplanes.

Once infection has taken place and its development gone unchecked, spread in transit generally occurs by the contact of a moldy or wet lesion with a healthy product, or as the old saying goes, one rotten apple in a barrel spoils all the rest.

Oftentimes, the development of one type of decay leads to the onset of other decays; this is often the case when secondary saprophytic or facultative organisms follow wholly parasitic organisms or primary causal agents. Occasionally, one organism provides the necrotic or dead areas of the skin for the entrance of other parasitic organisms. An example is bacterial soft rot *(Erwinia carotovora)* or fusarium rot (*Fusarium* ssp.) following late blight of potatoes *(Phytophthora infestans)*. These complications often make the identification of the direct causal agent difficult.

CONTROL OF DECAY

The responsibility for the safe delivery of such perishable items as fresh fruits and vegetables rests with everyone from the grower to the eventual receiver of the products. The same producers and handlers naturally stand to profit from reduced decay during marketing. The control of decay of these commodities may be based on the weak points of the infection process or the life cycle of the organism.

The principal means of control are to eliminate, kill, hold in check, or reduce numbers of, the pathogen. On the other hand, the commodity may be protected by making it less susceptible; for example, by altering its environment during transit and storage. Whatever the method, the aim is to shift the balance in favor of the host at the expense of the pathogen.

Decay control begins in the field or orchard with preharvest treatments and continues through harvest, storage, transit, and marketing. No step can be neglected in the effort to prevent decay from reducing the quality and quantity of fruits and vegetables available to the consumer.

Preharvest Control Measures

1. Removal and destruction of plant debris that may harbor plant pathogens. An example is the sanitation procedure of destroying piles of cull potatoes which can serve as a source of the primary inoculum of late blight of potatoes *(Phytophthora infestans)*.

2. The use of certified seed or plant stock known to be free of disease or those varieties that are disease and insect resistant. Included are those that are completely immune and those that resist fungus or bacterial infections and insect injury.

3. The timely and judicious field spraying of the various fruit and vegetable crops with proper chemicals to kill the load of inoculum that may be present and thereby reduce the incidence of infection.

Measures Applied at Harvest

Produce should be harvested at the proper stage of maturity, with care to avoid bruising. It is also most important that fruits and vegetables be carefully graded to eliminate material that may harbor diseases or insects, in order to prevent spread or initiation of new infections in storage or in transit.

Postharvest Measures

The wide variety of practices is determined by the nature of the produce, its susceptibility to decay, the length of the storage period, and the conditions under which the commodity is to be marketed.

A. Control Temperatures

1. *Refrigeration.* One of the principal methods in the control of decay before and during transit is by means of low temperatures. The aim is to reduce temperatures to a point where the growth of most plant pathogens is retarded (usually below 40 F), at the same time delaying the ripening of the produce. However, some fruits, especially those of subtropical or tropical origin such as bananas, mangos, and avocados, are quite susceptible to temperatures below 50 F and may be injured if held below that point for long periods. Tomatoes are particularly subject to decay if held at 40 F for more than three days.

The proper maintenance of quality and control of decay require refrigeration equipment to provide temperatures suitable to the commodity and efficient distribution of cooled air. For this purpose, storage rooms, rail cars, and trucks should be heavily insulated, and equipped with either mechanical refrigerating units with fans or ice bunkers with fans. These must be adequate for the space to be cooled and the Btu-releasing potential of the commodities.

Removal of field heat as rapidly as possible from harvested produce delays ripening and prevents the development of incipient field infections. Such commodities as peaches, celery, and asparagus are precooled as soon after harvest as possible, by immersion in, or spraying with, water held at 32-35 F (hydrocooling) before they are loaded into cars or trucks. Some produce is loaded, then cooled rapidly by top icing or by circulating cold air through the load by means of fans. Harvested commodities may also be precooled by placing them directly into the storage rooms held at low temperatures in which cold air is circulated. The cooling of head lettuce under vacuum has become standard practice in the West.

2. *Curing.* Warm temperatures are a means of reducing decay of some commodities. It has been shown that temperatures of 65-70 F at high relative humidity, enable potatoes to heal rapidly and thus resist the entrance of such pathogens as *Erwinia carotovora* (soft rot of potato). Likewise for sweet potatoes, temperatures of 80-85 F are effective in enabling this root to form a protective layer of wound cork and seal off invasions by *Rhizopus* spp.

B. Chemical Toxicants

These materials are applied in the form of a wash, dust, or gas. They must retard or kill pathogenic organisms without injuring the treated material and without leaving an objectionable residue. Their effectiveness is limited to surface loads of inoculum and to young incipient infections that have not penetrated deeply.

Sodium orthophenylphenate is used as a dip or in hydrocooling water to reduce decay of citrus fruits, mostly oranges and grapefruit.

Hypochlorites and free chlorine at 100-120 ppm reduce numbers of bacteria and fungi when used in precooling or wash baths.

Copper sulphate in a paste form is sometimes applied to watermelon stems for control of stem-end rot *(Diplodia natalensis)*.

Gassing or fumigating produce with adequate supplementary equipment to circulate the gas has the advantage of uniform dispersal of the toxicant in a load when in a tightly confined space as in storage rooms or in a rail car. The principal fumigant used on grapes in California is sulphur dioxide. Storage rooms and rail cars are degassed after treatment. The usual treatment for grapes to be stored is to apply the SO_2 to unrefrigerated grapes at a concentration of 1% for 20-25 min followed by the application of 0.25% for the same period of time in the cold storage rooms at 7 to 10 day intervals. In the case of grapes shipped directly to a market or where grapes have been in storage before shipment, fumigation is done in a rail car. The usual procedure is to release into the car 3-5 lb liquid SO_2 from cylinders heated in hot water to form the gas. In cars cooled by ice, supplemental fans are necessary to distribute the gas evenly because the standard car fans circulate the gas over the ice which absorbs it. Mechanically refrigerated cars distribute the gas evenly but the doors must be opened after fumigation to de-gas the car.

Nitrogen trioxide gas applied to citrus is effective against spores of fungi on the surface of the fruit but not against mycelium protected by the rind.

Ethylene and proplyene oxide are used on dried fruits and spices but not on fresh fruits and vegetables.

Formaldehyde gas is used against pathogens adhering to walls, floors, and crates in empty sweet-potato storage rooms.

All chemicals used on fresh fruits and vegetables, as on any food, must be approved by the Food and Drug Administration. This agency establishes a residue tolerance for chemicals after their safety has been proved.

C. Controlled Atmosphere Storage

While most methods of disease control center around a direct attack on the causal agents, in controlled atmosphere storage, the modification of the environment may not only result in favorable conditions for the storage of the commodity but also be a method of controlling to some extent pathogenic organisms that may be present. For example, it has been shown that carbon dioxide above the normal levels found in the air at standard temperatures and pressures, with a concomitant reduction of oxygen (ratio of 5% CO_2 to 2% O_2) acts to retard ripening and the development of decay organisms. Different fruits and even different varieties of the same fruit require different atmospheres to maintain optimum quality. This type of storage is effective on certain varieties of apples and pears harvested in the fall and may extend their storage life into the following spring with relatively little loss in quality.

D. Packing

The package in which fruits are shipped can serve as a modified cushion; therefore, emphasis is being placed on cell-type cartons to prevent contact bruises in the case of premium grade apples, pears, apricots, and plums. Chemically impregnated liners and wraps are used for specific fruit. Diphenyl impregnated into box liners or fruit wraps may volatilize at both refrigeration and room temperatures. By its fungistatic action, it is effective against species of *Penicillium* and the fungi causing stem-end rot *(Phomopsis citri* and *Diplodia natalensis)* on citrus. Copper compounds, while not effective in a volatile form, are impregnated into wraps used for pears and prevent contact infections of *Botrytis.*

The popularity of polyethylene films for packaging and preventing moisture loss in fresh fruits and vegetables influences the overall problem of decay control. By virtue of the nature of these films the normal gas and water vapor concentrations in the immediate environment of produce can be altered. For instance, the modification of the atmosphere rapidly established in a poly pack of sweet cherries markedly reduces decay in shipping. In the case of bananas, a polyethylene wrap reduces the incidence of scarring, bruising, and resulting decay during the trip from plantation to market. However, polyethylene is not suitable for all commodities and causes off-flavors in some fruits, for example strawberries, peaches, and nectarines. Another disadvantage is the fact that produce packed in film may cool slowly. Finally, the high relative humidity maintained in a semipermeable film can actually increase the amount of decay in such items as Italian prunes. Decay of some commodities, onions and citrus for example, is reduced if the polyethylene bags are perforated.

SUMMARY

There is no one easy method to follow for the control of decay in fruits and vegetables. In some cases, the attack begins before the crop is planted. Good production practices, such as preharvest treatments to reduce insect damage and the numbers of pathogenic organisms that cause decay, will help minimize actual spoilage during marketing. Careful harvesting, handling, and packing prevent injuries that serve as targets for the ubiquitous pathogens. Postharvest treatments, especially suitable refrigeration, help insure a good return on the investment put into the produce and supply consumers with an abundance of high quality fruits and vegetables at reasonable cost.

COTTON MADE RESISTANT TO MICROBIOLOGICAL DETERIORATION USING FORMIC ACID COLLOID OF METHYLOLMELAMINE

W. Norbert Berard, Ethel K. Leonard, and Wilson A. Reeves

Southern Regional Research Laboratory (Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture), New Orleans, Louisiana

It is well known that cotton will deteriorate rapidly when exposed to microbiological and photochemical degradation, and considerable research has been devoted to solve these problems. A number of additive type fungicides, which have been developed in recent years, have extended the useful life of cotton products in many end uses. However, these chemicals have drawbacks such as lack of durability and unacceptable coloration of the fabric.

Another approach to this problem has been through chemical modification of the cellulose by reaction with various organic chemicals. The material retains completely the appearance of cotton but acquires new properties which have been of interest to the textile industry. Investigations by Goldthwait *et al.* (1946) and Cooper *et al.* (1952) have shown that cotton chemically modified by partial acetylation is resistant to rotting and mildewing and withstands soil-burial tests for much longer periods than does cotton treated with additive type fungicides. Partial acetylation is the process of attaching acetyl radicals onto cellulose molecules. Figure 1 is a schematic representation of a substituted cellobiose unit which illustrates a degree of substitution of unity (D.S. of 1.0).

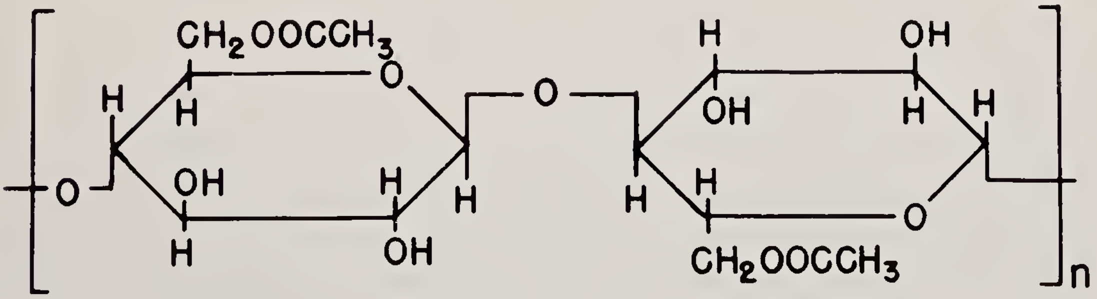

Fig. 1. Partially acetylated cellulose.

Another chemical modification that has been investigated is cyanoethylated cotton, a product of the reaction of acrylonitrile with cotton to yield a cyanoethylcellulose ether. The studies of Compton *et al.* (1953) have shown that cyanoethylated cotton can withstand soil burial exposure for extended periods without significant loss in strength. The schematic representation for cyanoethylated cotton with D.S. of 0.5 is shown in Fig. 2. Acrylonitrile polymerized in cotton fabrics is also an effective means of protecting cotton from microorganism attack (Gottlieb and Jeffries, 1957; Haydel *et al.*, 1957).

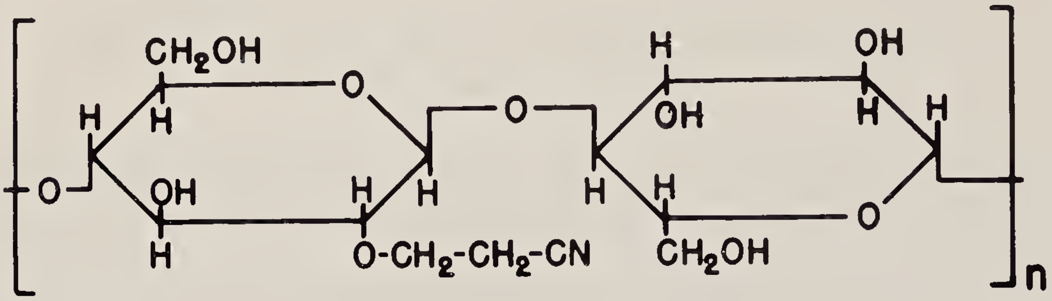

Fig. 2. Partially cyanoethylated cellulose.

Other chemical modifications of cellulose have been prepared which show some resistance to microbiological attack. Studies by Guthrie (1947) demonstrated that 2-aminoethylsulfuric acid reacts with cellulose under alkaline conditions. Upon subsequent treatment with a mercury salt, the aminized fabric showed rot resistance in a soil-burial test. The amino groups serve as effective means for attachment of various metals.

Partial carboxymethylation of cellulose reported by Reid and Daul (1947) does not protect it from microbiological attack. However, addition of various metals to form insoluble salts of carboxymethylcellulose imparts considerable resistance to such attack.

Investigations by Frick *et al.* (1957) have shown that cellulose can be reacted with acrylamide to give modified cotton having good fabric properties. Carbamoylethylated cotton as prepared showed a slight degree of rot resistance, but this could probably be increased with a higher degree of substitution.

Recent studies on the reaction of chloromethylphosphonic acid with cellulose, described by Hobart *et al.* (1959) showed that phosphonomethylated cotton in the copper salt form was superior in rot resistance to the sodium and ammonium salt forms and to untreated cotton. However, the degree of rot resistance is not comparable to that imparted by commercial rot-resistant treatments.

In all of the above work on chemically modified cottons, partially acetylated and cyanoethylated cottons had excellent resistance to rot. Due to the cost of these products they have not been utilized commercially as rot resistant cotton fabrics. The other chemically modified cottons depend upon a subsequent reaction with metal salts to produce rot resistance, equivalent to that obtained with additive type fungicides now available commercially.

In considering all of the above factors it was evident that a durable, colorless, and inexpensive rot-resistant treatment that would be easy to apply by textile finishing companies, was needed for cotton. The use of aminoplast resins seemed to offer good possibilities, as previous investigations (Nute, 1945; White and Siu, 1947) with melamine formaldehyde have demonstrated that resin-impregnated cotton fabrics were resistant to microbiological deterioration when subjected to soil-burial tests. However, these results were not as good as those obtained by partial acetylation or cyanoethylation. In all of the above work, the melamine formaldehyde and other aminoplast resins were applied by the "conventional process." "Conventional process" means the application of an aqueous system of essentially a monomeric-resin former and a latent acid catalyst to a textile followed by a dry and a cure at elevated temperatures. Recent studies at this laboratory have shown that an acid colloid of trimethylolmelamine can make cotton quite resistant to degradation in the soil bed (Berard *et al.*, 1959).

The purpose of the present paper is to report additional studies on application techniques of methylolmelamine resins to cotton, and to reveal a practical method for producing cotton fabric with excellent rot and good weather resistance. The structural formula for trimethylolmelamine is shown in Fig. 3. This method is based on the use of a formic acid colloid of methylolmelamine (Berard *et al.*, 1959). The method of application of the colloid is essentially the same as the conventional method for resin finishing; that is, pad, dry, and cure. The main difference is the use of formic acid in the treating solution to serve as the catalyst and most important to produce a colloid with the trimethylolmelamine. The term "acid colloid" as used in this paper refers to methylolmelamine molecules and polymers of methylolmelamine which are associated and stabilized by polar acid groups. All of these colloids possess light-scattering properties.

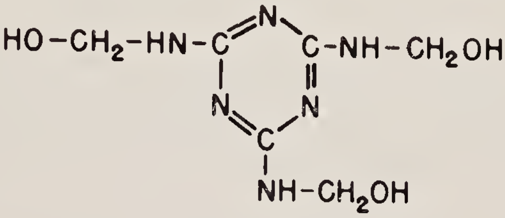

Fig. 3. Trimethylolmelamine.

Acid colloids of aminoplast resin formers have been used before, but according to the way they were produced and applied to textiles, they did not impart much rot resistance. Nute (1949) has shown that colloids of methylolmelamine are unique textile finishing materials—they produce excellent shrinkage control and durable stiffness, with no loss in tensile strength. In the earlier studies the colloids were permitted to age, often as much as 24 hr, thus allowing rather large polymers or colloidal particles to form and deposit on the surface of the cotton fiber. Cooke (1954) observed that a cotton fabric impregnated with an aged acid colloid of methylolmelamine completely deteriorated when exposed to a 2-week soil-burial test. This was a much shorter deterioration time than that observed when the resin was applied by the conventional method.

MATERIALS AND METHODS

Fabric Description

Most of the experimental work was done with a desized, scoured and bleached 80 x 80 cotton print cloth weighing approximately 3.12 oz/yd^2. Other fabrics used included a desized and scoured 48 x 48 cotton sheeting, and a scoured 8-oz cotton army duck.

Chemicals Used

The N-methylol compounds used were commercial products sold under various trade names. The compounds were unmethylated methylolmelamine (Resloom HP*, Aerotex Resin UM Special*). Formic acid (90% purity) and lactic acid (85% purity) were commercially available products.

Processing Procedure

In each case the resin-forming monomer, and crosslinking agent, were first dissolved in water at room temperature, and then the acid catalyst was added. In most cases the solutions were used within 10 min; in a few instances, where indicated, the solution containing formic acid was allowed to stand one or more hours after the acid was added before it was used. Samples of cotton fabric were impregnated on a laboratory padder, dried 4 min at 80 C, and cured for the same length of time in a forced draft oven, at the temperatures indicated below. Following the curing step, samples were washed at 100 F with Tide* as the detergent in a typical agitator-type home automatic machine on the modern-fabric cycle. The cloth samples were then tumbled dry in a gas-heated home dryer.

Test Methods

The following properties were determined by the usual ASTM procedures: breaking strength, strip method (1a) (except the strips of print cloth were raveled to 80 threads, the sheeting to 48 threads, and the army duck to 55 threads), and soil burial (1b). Samples were leached with water according to Fed. Spec. CCC-T-191b, Method 5830.

Weather exposure tests were conducted in New Orleans, Louisiana, in an unshaded location, with the samples exposed on open-backed wooden rack facing south at an angle of 45° from the vertical and at a minimum distance of 30 in. above the ground.

RESULTS

Effect of Aging of Colloids on Rot Resistance

Previous work at this laboratory (Berard *et al.*, 1959) on application techniques of methylolmelamine resins to cotton revealed that cotton fabric with outstanding rot resistance and good weather resistance could be produced. Print cloth samples were treated with solutions containing 17% methylolmelamine and 20% formic acid (a molar ratio of about 1:5), which gave 12% resin add-ons, to explore the value of formic acid colloids and the effects of aging colloid solutions. The fabric was impregnated immediately (within 10 min) after preparing the colloidal solution, and then at 2-, 4-, 5-, 6-, and 7-hour intervals. Table 1 shows breaking strengths in pounds of the different samples as treated and at the indicated periods of soil burial. The sample treated with freshly prepared colloid solution retained all of its original strength after 21 weeks of soil burial, and the fabric impregnated with the solution aged for 2 hr had a breaking strength of 26.0 lb, compared to its original strength of 33.3 lb, for the same period of exposure. The rest of the fabrics showed a decreasing resistance as the colloid solution was allowed to age. These results demonstrate that freshly prepared acid colloid resin solutions (and those aged less than 2 hr) impart to cotton fabrics excellent resistance to microbiological deterioration. Fabrics treated with the aged colloids offered much less resistance to microorganisms. In previous studies (Berard *et al.*, 1959), fabric treated with trimethylolmelamine by the conventional process to contain 12% resin add-on, had completely deteriorated after 6-weeks soil burial.

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*Use of a company and/or product named by the Department does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

Table 1. Effect of Aging of Colloids on Rot Resistance

Age of colloid used to produce 12% resin in finished fabric	Breaking strength in pounds, before and after soil burial						
	Orig.	2 Wks.	4 Wks.	6 Wks.	12 Wks.	18 Wks.	21 Wks.
Freshly prepared	32.3	36.1	39.4	34.6	40.9	40.9	32.6
2 Hours	33.3	38.0	39.0	36.5	38.1	30.6	26.0
4 Hours	36.0	36.5	40.5	36.7	22.5	7.4	-
5 Hours	38.4	39.9	41.1	28.9	14.4	9.3	-
6 Hours	37.7	39.2	38.4	28.8	7.8	2.7	-
7 Hours	39.0	37.8	34.0	24.4	6.0	-	-

Influence of Colloid Add-on upon Rot Resistance of Treated Fabric

As the above work had been confined to using 12% add-ons of the trimethylolmelamine resin, it seemed desirable to determine if lower concentrations of resin would afford similar resistance to microbial attack. Therefore, print cloth was treated to contain from 3% to 12% resin add-on. For the 12% resin add-on, the fabric was treated with a solution containing 17% trimethylolmelamine and 20% formic acid. To obtain the lower resin add-ons, more dilute solutions of trimethylolmelamine were used, but the concentration of formic acid was held constant. For example, 3% resin add-on was obtained by using a solution containing 3.4% trimethylolmelamine and 20% formic acid.

Table 2 gives the breaking strengths in pounds of the samples with the various resin add-ons as treated and after the indicated periods of soil burial. The sample with 12% resin retained all of its breaking strength after 18-weeks soil burial, and the sample with 10% resin add-on had retained approximately 2/3 of its strength for the same period of exposure. The samples containing still lower resin add-ons retained even less strength which was directly related to resin add-on. The sample with 3% resin was completely deteriorated in 2 weeks of soil burial. The results show that excellent resistance to microorganisms was obtained when the cloth was treated with freshly prepared acid colloid solutions so as to impart 10–12% resin add-on.

It should be noted in the above two tables that there is an increase in breaking strengths of the treated fabrics after various periods of soil burial. This phenomenon cannot be attributed to fabric shrinkage and thereby breaking of more warp yarns, as all samples were stripped to a specific thread count before the soil-burial tests. Buras and coworkers (1954) report that a small gain in strength

Table 2. Influence of Add-on of Formic Acid Colloid Upon Degree of Rot Resistance

Resin Add-on in %	Breaking strength in pounds before and after soil burial							
	Orig.	2 Wks.	4 Wks.	6 Wks.	9 Wks.	12 Wks.	15 Wks.	18 Wks.
12	26.6	37.5	36.0	38.3	38.3	40.6	38.5	39.4
10	31.2	31.8	35.3	33.8	36.3	22.4	21.4	21.6
8	33.4	35.4	10.9	8.8	5.8	1.3	1.1	-
5	34.3	11.8	2.1	-	-	-	-	-
3	35.1	-	-	-	-	-	-	-

is often observed in acetylated cotton during the early stages of soil-burial tests; this effect is paralleled in heat tests as well. It is evident that additional studies are needed before a satisfactory explanation can be given of these unusual increases in breaking strengths.

Effect of Curing Temperatures and Water Leaching on Rot Resistance of Acid Colloid — Finished Print Cloth

In the above work it was found that the freshly prepared formic acid colloid could be cured in print cloth adequately at 140 C to provide a high degree of resistance to microbiological attack. Therefore a study was made to establish the minimum curing temperature needed for optimum rot resistance. Print cloth samples were treated with an acid colloid solution containing 17% trimethylol-

Table 3. Effect of Curing Temperatures and Water Leaching on Rot Resistance of Acid Colloid — Finished Print Cloth

12% resin in finished fabric *	Cure Temp. (°C)	Breaking strength in pounds, before and after soil burial					
		Orig.	2 Wks.	4 Wks.	6 Wks.	12 Wks.	15 Wks.
Unleached	-	42.9	45.5	48.5	35.2	2.3	-
Leached	-	35.3	44.8	42.9	27.0	2.1	-
Unleached	80	40.4	44.1	46.6	49.1	25.8	11.2
Leached	80	36.7	42.3	48.0	51.3	18.7	9.8
Unleached	100	31.9	39.7	43.0	43.8	26.7	26.4
Leached	100	31.9	38.9	42.0	44.0	29.2	22.6
Unleached	120	32.3	40.5	41.3	44.6	28.0	22.9
Leached	120	34.0	39.8	41.2	42.8	42.0	31.2
Unleached	140	31.5	36.2	40.8	38.4	39.4	35.7
Leached	140	33.9	32.4	41.8	42.2	33.6	40.4
Untreated, control	-	51.4	-	-	-	-	-

*All samples dried 4 min at 80 C.

melamine and 20% formic acid to give 12% resin add-ons and dried 4 min at 80 C. One sample was not cured while other samples were cured at 80, 100, 120, and 140 C for 4 min.

To determine the durability of this finish, part of each of the above samples was leached in water at a rate of about 5 changes/hr for a period of 24 hr.

Table 3 shows the breaking strengths in pounds of the different samples as treated, and after leaching, and at the indicated periods of soil burial. After 15-weeks soil burial, the fabric cured at 140 C, unleached or leached, had retained all of its strength. The fabrics cured at the lower temperatures retained less breaking strength. Samples cured at the lowest temperatures retained the least strength. The sample not cured completely deteriorated in the 15-weeks soil burial. The leaching had practically no effect upon the samples as most breaking strengths were about the same as the unleached samples. These results demonstrate that a cure temperature of at least 140 C is necessary to produce excellent rot-resistant cotton, and also that the finish is durable and not affected by a severe water-leaching test.

Preparation of Stable Acid Colloids of Trimethylolmelamine by Use of Other Acids

Since stable acid colloids of trimethylolmelamine had been obtained with formic acid, investigations were conducted to determine if glycolic and lactic acids would also produce stable colloids with trimethylolmelamine, and if such

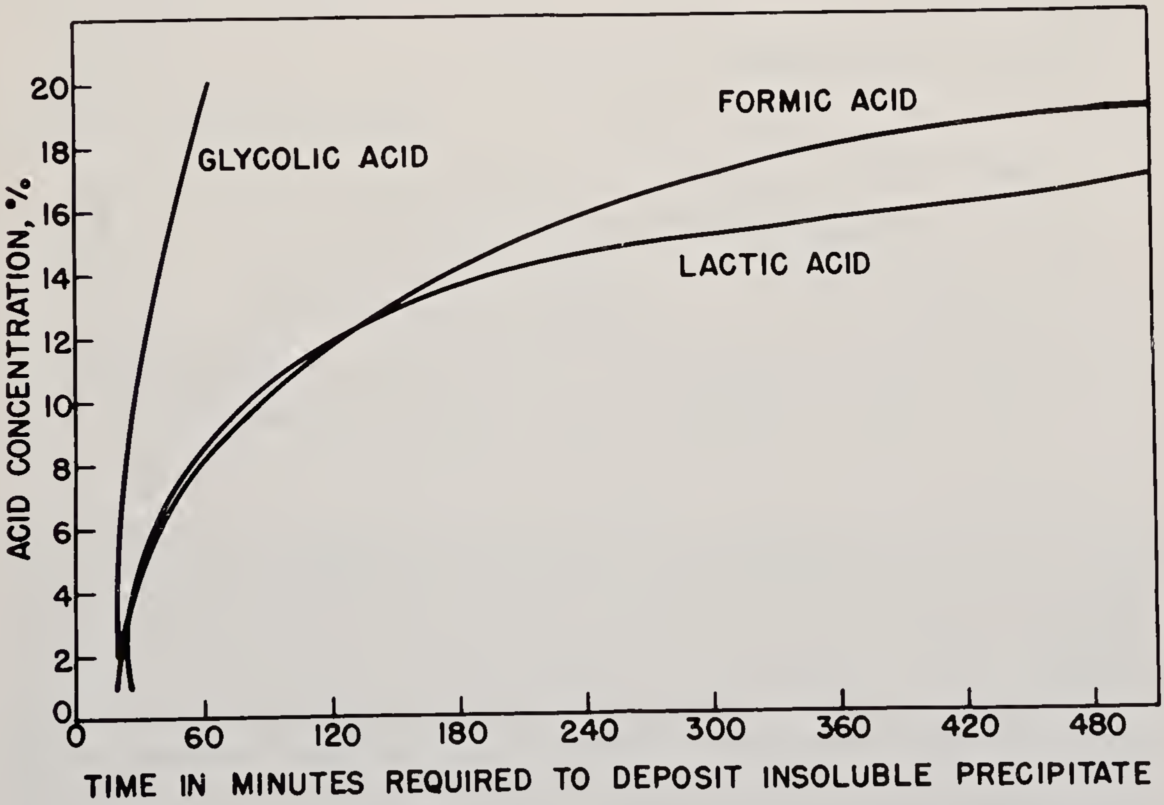


Fig. 4. Time of stability of the colloidal solutions with varying amounts of formic, lactic, and glycolic acids.

colloids could be used to impart the degree of rot resistance obtained with formic acid. A series of solutions were made in which the methylolmelamine concentration was held constant at 17% while the amount of formic, lactic, or glycolic acids was varied up to 20% of the weight of the total solution. The time required for a precipitate or gel to occur was noted.

Figure 4 gives the time of stability of the colloidal solutions of the three acids, as judged by the appearance of a visible precipitate. The solutions with 4% or less acid began to deposit a precipitate within 25 min. At these low concentrations, the three acids act primarily as polymerization catalysts. Higher concentrations of formic and lactic acids have a stabilizing effect and also act as polymerization catalysts. The colloid solutions containing 20% formic acid and 18% lactic acid were stable for at least 8 hr, but did form clear gels sometime before 24 hr. The glycolic acid was not effective in producing a stable colloid even at 20% concentration, as an insoluble precipitate formed in just over one hour. Obviously, the larger amounts of formic and lactic acids have a marked stabilizing effect on the colloid.

Rot Resistance of Print Cloth Finished With Formic and Lactic Acid Colloids of Trimethylolmelamine

As the stability curves for the formic and lactic acids were very similar, it seemed desirable to compare the microbiological resistance of fabric finished with these two colloidal solutions. Print cloth samples were treated with the formic acid and lactic acid colloids of trimethylolmelamine to give approximately 12% resin add-ons. The samples were dried 4 min at 80 C and cured for the same length of time at 140 C.

Table 4 gives the breaking strengths in pounds of the samples as treated and after the indicated periods of soil burial. The sample finished with the formic acid colloid had retained all of its original breaking strength after 15-weeks soil burial, while the sample finished with the lactic acid colloid had retained a little less than half of its original breaking strength. This would indicate that the formic acid colloid imparts higher resistance to microbial attack than the lactic acid colloid.

Table 4. Rot Resistance of Print Cloth Finished With Formic and Lactic Acid Colloids of Trimethylolmelamine

Acid colloid used, 12% resin in finished fabric	Breaking strength in pounds, before and after soil burial					
	Orig.	2 Wks.	4 Wks.	6 Wks.	12 Wks.	15 Wks.
Formic Acid	31.5	36.2	40.8	38.4	39.4	35.7
Lactic Acid	39.8	44.2	31.1	51.0	28.3	15.6

Viscosity measurements were made, using a capillary tube, on the freshly prepared formic acid and lactic acid colloids, and after aging for various periods of time. The viscosity of the lactic acid colloid increased at a more rapid rate than the formic acid colloid. The higher viscosity of the lactic acid colloid at the time of application probably caused more resin deposition on the fiber surface, similar to the deposition of resin previously reported for aged colloids, and afforded less resistance to microbial attack.

Weather Resistance of Cotton Fabric Treated with Formic Acid Colloid of Trimethylolmelamine

As previous investigations on the aged acid colloid of methylolmelamine had not included weather exposure evaluations, it seemed desirable to evaluate fabric finished with the freshly prepared formic acid colloid. Samples of 8-oz duck and 48 × 48 sheeting were treated with acid colloid solutions to give approximately 12% and 6% resin add-ons for each fabric.

Figure 5 shows the breaking strengths obtained on the treated 8-oz duck samples and untreated control after weathering for periods up to 12 months. The two treated fabrics possessed very similar strength retentions and had retained all of their original breaking strengths after 12-months exposure. The curves indicate that the sample containing 12% resin add-on had a slight advantage over the fabric containing 6% resin add-on. This is probably due to the fact that duck fabrics retain moisture for a longer period of time than lightweight cotton fabrics, and the higher resin add-on afforded more resistance to microbiological attack. Although the untreated control fabric had a higher initial breaking strength, after 6–7 months of exposure the breaking strength was about equal to that of the treated fabrics. After 12-months exposure the strength of the control fabric was much lower than that of the acid colloid – finished samples.

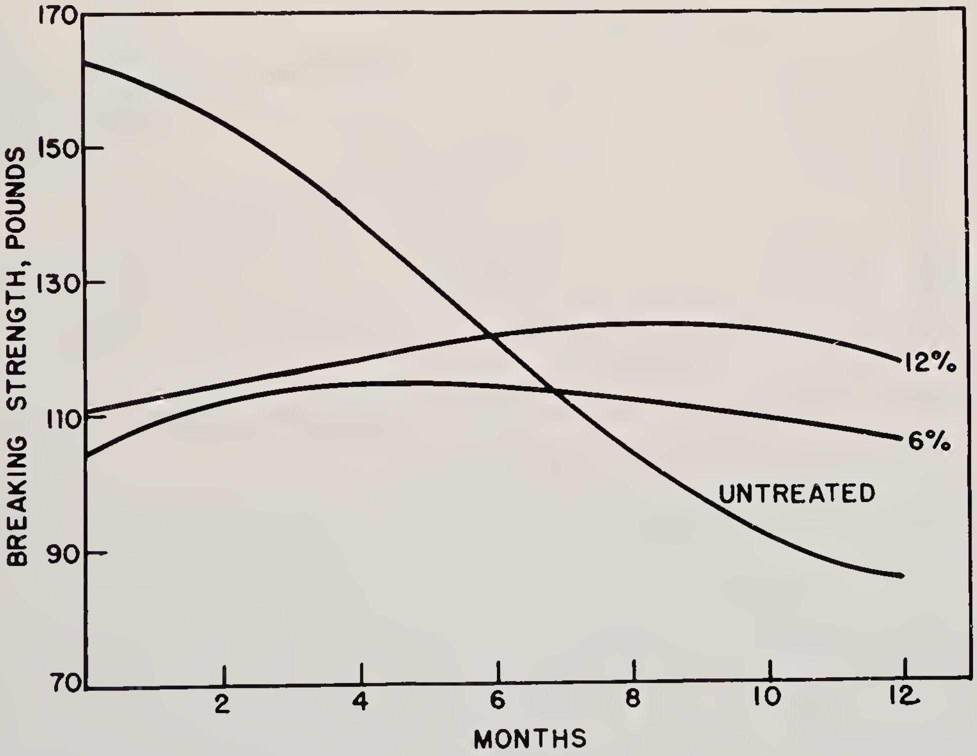


Fig. 5. Weather resistance of 8-oz duck treated with formic acid colloid of methylolmelamine at 12% and 6% resin add-ons, and untreated control.

Figure 6 shows the breaking strengths of the acid colloid – treated 48×48 sheeting, and untreated control, after weather exposure for periods up to 12 months. The breaking strengths for the 12% and 6% resin add-on samples are essentially the same for the various exposure periods, with practically no loss of strength after 12-months weathering. For lighter weight fabrics which dry out relatively fast after a heavy dew or rain, these results would indicate that the 6% resin add-on would be satisfactory as a weather-resistant finish. The untreated sheeting had a higher initial breaking strength, but was about equal in strength to the acid colloid – finished fabrics at the 4-month period. After 12 months the untreated fabric had a much lower breaking strength than the treated fabrics.

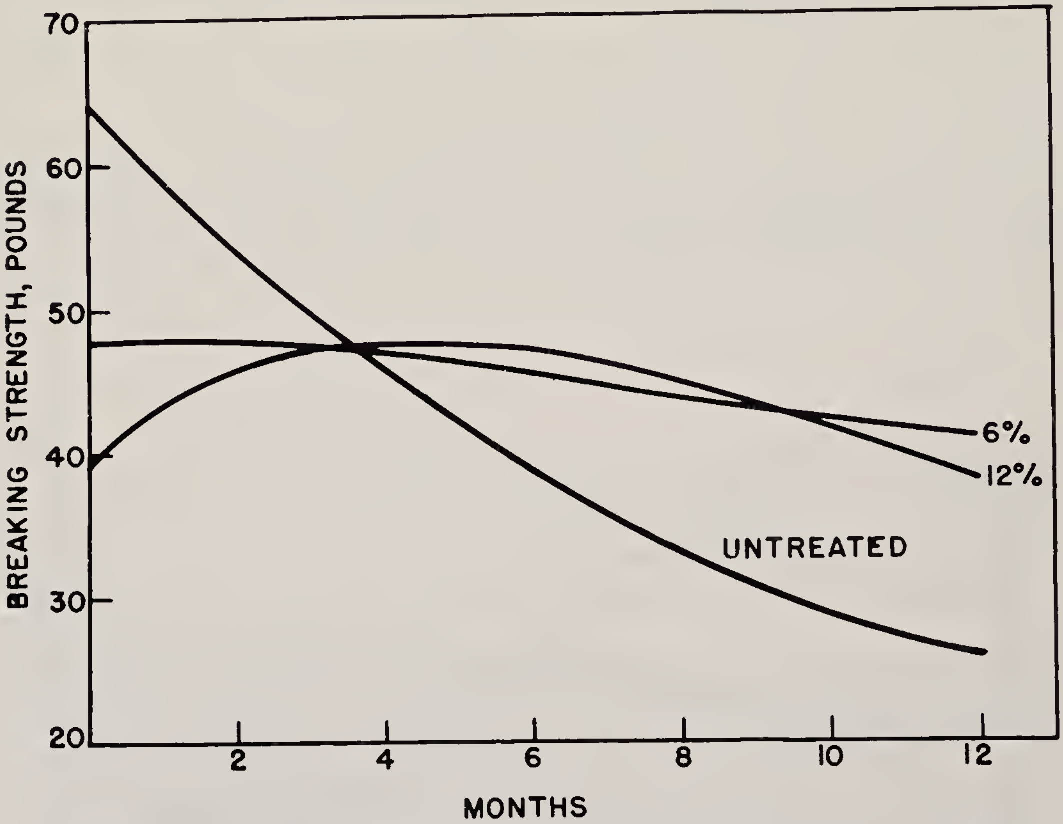
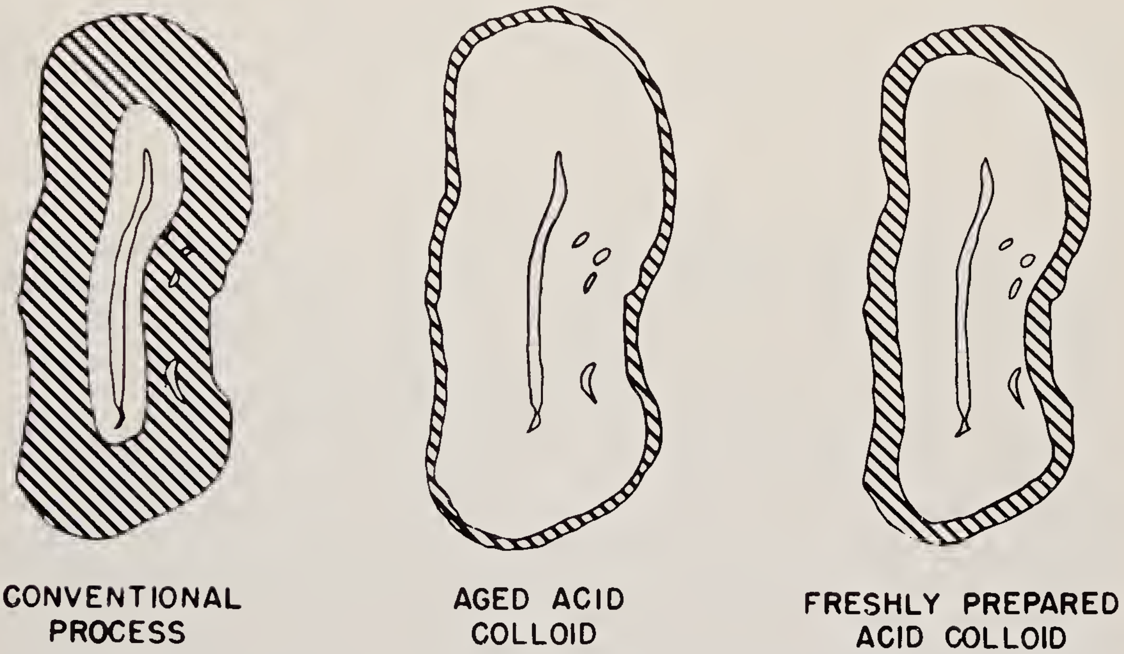


Fig. 6. Weather resistance of 48x48 sheeting treated with formic acid colloid of methylolmelamine at 12% and 6% resin add-on, and untreated control.

Microscopic Examination of Acid Colloid — Finished Cotton

Microscopic studies were made on fibers treated with the freshly prepared formic acid colloid of methylolmelamine, with aged acid colloid, and by the conventional technique, by treating ultrathin sections of these fibers with cupriethylenediamine hydroxide. Figure 7 shows a schematic drawing of cross sections of these fibers with the location of resin deposition. The cotton fiber treated with the fresh acid colloid had the resin deposited primarily in the outer portion of the cell wall. The innermost section of the fiber responded to cupriethylenediamine hydroxide by inhomogeneous solution in a manner similar to that observed for cotton having small amounts of bound formaldehyde. Examination of the aged acid colloid – treated fibers showed the resin was deposited only on the surface of the fiber. The conventionally processed fibers were found to be uniformly penetrated by the resin.


CONVENTIONAL PROCESS

AGED ACID COLLOID

FRESHLY PREPARED ACID COLLOID

Fig. 7. Schematic drawing of cross sections of cotton fibers showing location of resin deposition.

DISCUSSION

Cotton fabric was made rot- and weather-resistant by treating with a freshly prepared formic acid colloid of methylolmelamine. The method of finishing consisted of the usual pad, dry, and cure procedure. Best rot resistance was obtained by using acid colloids within 2 hr after preparation. Cotton print cloth finished with 12% resin imparted by use of a fresh acid colloid retained 100% strength after 21 weeks of soil burial. These results were substantially better than those reported for the same trimethylolmelamine applied by the conventional technique, and is much better than is known for additive-type fungicides. Previous work with similar acid colloids, aged at least 12 hr, and as long as 24 hr, did not impart rot resistance to cotton fabric.

Different curing temperatures had a marked effect on the rot resistance of the fabric. The results showed that a cure temperature of at least 140 C is required to produce excellent rot resistance. A severe water leaching test had practically no effect on the resistance to microorganisms in soil-burial tests, indicating excellent durability of the acid colloid finish.

Stable acid colloids were obtained when the colloidal solutions contained about 1 mole of trimethylolmelamine to 5 moles of formic acid. Colloidal solutions prepared with lactic and glycolic acids were not as effective for this purpose as formic acid. Viscosity measurements of a formic acid colloid and a lactic acid colloid, as measured through a capillary tube, showed that the lactic acid colloid had a higher viscosity than the formic acid colloid. This probably caused more of the resin to be deposited on the fiber surface, thereby imparting less resistance to microbial attack.

The outdoor weathering resistance of fabric was improved by the acid colloid finish. The results show that two different weights of cotton fabric, each at two levels of resin add-ons, retained all of their breaking strengths after 12-months weather exposure, while the untreated samples lost 50% or more of their breaking strengths for the same period of exposure.

Microscopic observations on fibers treated with the formic acid colloid of methylolmelamine showed that the resin was deposited primarily in the outer portion of the cell wall of the cotton fiber—which portion did not respond to swelling in cupriethylenediamine hydroxide; the innermost section of the fiber showing inhomogeneous solution in this reagent in a manner similar to that observed for cotton having small amounts of bound formaldehyde. Fibers treated with an aged acid colloid had a coating of resin on the surface of the fiber, and fibers treated by the conventional technique were quite uniformly penetrated by the resin.

It has been suggested that the microbiological degradation of cotton can be prevented by an inert physical barrier which interrupts the contact between the organisms and the cellulosic substrate. According to this mechanism, any cracks in the barrier or resin film should permit the fungi to penetrate to the cellulose, and cause deterioration of the cotton. To test this mechanism, print cloth containing 12% acid colloid resin was stretched through a number of cycles to 50% of the fabric breaking-load to produce ruptures in the "resin film," and then subjected to soil-burial tests. The fabric still maintained excellent rot-resistant properties, indicating that the acid colloid finish does not act as an inert physical barrier but rather is a chemical barrier to protect the cotton from microbiological attack.

SUMMARY

Cotton fabric has been made rot- and weather-resistant by treating with a freshly prepared formic acid colloid of methylolmelamine. Previous investigations have shown that acid colloids allowed to age at least 12 hr, and as long as 24 hr, do not impart much resistance to microbiological deterioration. The method of application of the freshly prepared acid colloid is essentially the same as conventional procedures used for resin finishing, that is, pad, dry, and cure. The main difference is the use of formic acid in the treating solution to produce a colloid.

Cotton fabric finished with the acid colloid of methylolmelamine at about 12% resin add-on had excellent resistance to microorganisms compared to cloth finished with methylolmelamine in the conventional manner at the same level of resin add-on. A curing temperature of at least 140 C is required to obtain excellent resistance to microbiological attack.

In microscopic studies of fibers treated with the formic acid colloid of methylolmelamine made by treating ultrathin sections with cupriethylenediamine hydroxide, the resin was found deposited primarily in the outer portion of the cell wall of the cotton fiber, which portion did not swell in the reagent; the innermost

section of the fiber responded to cupriethylenediamine hydroxide by inhomogeneous solution in a manner similar to that observed for cotton having small amounts of bound formaldehyde.

ACKNOWLEDGMENTS

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THE BIODEGRADABILITY OF ALKYLBENZENE SULFONATES

J. F. Nelson, R. E. McKinney,* J. H. McAteer,
and M. S. Konecky

Chemical Research Division, Esso Research and Engineering Company, Linden, New Jersey

The excellent acceptance of synthetic detergents (syndets) has led to a United States consumption that is now three times that of soap (Anonymous, 1959a). A rapid growth has also occurred in Europe. In 1958, syndets constituted nearly 90% of the total soap plus syndet consumption in Germany (Anonymous, 1959b). From frequent articles in the press and the literature, it is evident that this increase has been accompanied by problems such as foaming in sewage disposal plants and in the waters receiving the effluent from these plants. Frequent mention is made of several feet of foam covering portions of the waters in sewage plants, canals, and rivers in Europe (Anonymous *et al.*, 1959 and 1960). Only a few reports on the sewage foaming and related problems in the United States have been published, where the problem appears not to be widespread, as it is in many areas overseas.

There is considerable evidence that syndets are a contributory cause to the increase in foam, although other factors are undoubtedly involved in the problem. Certain synthetic detergents have been reported to be less readily destroyed than soap by the microorganisms that occur in sewage disposal plants. As a result, the concentration of surface active agents has increased in sewage disposal plants and in the plant effluents.

The present study was initiated to provide information on the effect of molecular structure on the biodegradability of synthetic detergents. The initial work has been limited to sodium alkylbenzene sulfonates, since syndets of this type enjoy a large market and they are the subject of much research on improving detergency by varying the size and structure of the alkyl group. Compounds of known side chain structure were synthesized and their biodegradability determined.

Domestic sewage is processed principally in two types of plants, one of which uses a trickling filter and the other an activated sludge which is continuously aerated. Since activated sludge plants are quite prevalent in the United States, our work was modeled around this type of operation. Glass aeration units containing sewage microorganisms were set up in the laboratory. These units were used to acclimate the sludge to the syndets and to provide effluent for a foam test which gave an indication of residual syndet.

Previous observers have shown that the syndet can disappear from solution in two ways. It can serve as food for the microorganisms, and according to the results of McGauhey and Klein (1959), Polkowski *et al.* (1959), and other investigators, it can also be absorbed on or associated with the sludge. For this reason we ran Warburg tests in which net oxygen consumption was determined as a measure of the proportion of the syndet being used as food. Although the results from the Warburg tests are not always as precise as those from other commonly used test procedures, they have the advantage of providing an indication of metabolism.

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*Present address: University of Kansas, Lawrence, Kansas.

EXPERIMENTAL METHODS

Equipment and Procedure

Activated sludge was grown in 2.5-L tubes (aeration units) containing 1.5 L of mixed liquor. Air at 10 psi was introduced through two jets at the bottom of the tubes. The volume of air was controlled to keep the activated sludge in suspension, but was not measured. It has been shown from previous studies that under these conditions sufficient oxygen is assured to meet the demands of the microorganisms.

The aeration units were operated on a fill and draw basis over a 24-hr cycle. The mixed liquor was aerated for 23 hr following which the air was shut off and the sludge permitted to settle for a period of 1 hr. After settling, 1 L of the clear supernatant liquor was poured off leaving the sludge in the remaining 500 ml. The withdrawn liquor was replaced with 1 L of synthetic sewage solution and aeration was again started. In a number of instances a small proportion of the activated sludge itself was withdrawn or wasted in order to induce more active bacterial growth. In such cases the amount withdrawn corresponded normally to about 100 ml/day of the total 1.5 L of mixed liquor.

Activated sludge seed was taken from a commercial sewage-treating plant. The bacterial sludge was grown on synthetic sewage having the following composition: nutrient broth, 125 mg/L; castile soap, 125 mg/L; sodium benzoate, 125 mg/L; and K_2HPO_4, 125 mg/L. The solvent in all cases was Cambridge, Massachusetts tap water. This synthetic sewage was fed to the activated sludge to give a concentration of about 375 mg/L.

At the start, the sludges were adapted to the sodium alkylbenzene sulfonates (ABS) by introducing the sulfonate first at a concentration of 5 mg/L with the synthetic sewage and then increasing the ABS load 5 mg/L per day for 10 days until 50 mg/L of ABS was being fed daily. After about 3- to 4-weeks feeding with ABS at the 50 mg rate, the first of a series of Warburg runs was made using the activated sludge.

Conventional Warburg equipment was used in the present work. Runs were made at intervals, usually, of 4 weeks. Mixed liquor from the aeration unit (100 ml) was permitted to settle so that the activated sludge assumed twice its original concentration. Ten ml of this concentrated sludge was placed in a 125 ml Warburg flask containing 1 ml of 10% KOH in the center receptacle to absorb the CO_2 produced. Two flasks were prepared for each sludge. To one flask 10 ml of water was added, while to the other 10 ml of a solution of the ABS (100 mg/L) was added. The ABS-free system was used as the control.

Oxygen uptake was determined by measuring the change in pressure in the constant-volume Warburg system over a period of 28 hr. The contents of the flask were then filtered through a membrane filter to remove all microorganisms. The filtrates were analyzed as described below.

Chemical Oxygen Demand (COD)

The dichromate chemical oxygen demand test, modified for use with Warburg filtrates, was employed to determine the over-all disappearance of oxidizable organic matter in both the aeration unit effluents at the time samples were taken for Warburg runs and the filtrates recovered from the control and fed Warburg flasks. In this test a 10 ml sample of aqueous solution is reacted with 5 ml of 0.25 N $K_2Cr_2O_7$ and 15 ml of concentrated H_2SO_4 containing 1 g Ag_2SO_4 per 75 ml of acid. After refluxing for two hours, the samples are cooled and titrated with 0.05 N ferrous ammonium sulfate to an end point with ferron indicator.

Methylene Blue Extraction (MB)

Extraction of the alkylbenzene sulfonic acid — methylene blue complex from an acidified aqueous solution by means of chloroform was employed to obtain a direct measure of the concentration of ABS. The method is essentially that previously described by Degens *et al.* (1953). The spectrometer used to determine the transmission at 640 mμ had been previously calibrated with a standard ABS. In all cases a blank sample was run simultaneously with the unknown. A methylene blue test was run on samples of the aeration unit effluent at the time of each Warburg run and on the filtrates recovered from the Warburg flasks.

Foam Test

Foam tests were carried out on samples of the aeration unit effluents at the time of each Warburg run. This test was devised specifically for the present study. Effluent (150 ml) is placed in a cylinder 7 cm in diameter and approximately 50 cm high. Air is introduced through a diffuser (medium porosity, sintered glass) at the bottom of the tube at a rate of 100 ml/min. After 5 min aeration the foam height is measured and recorded. Under these test conditions a typical commercial type ABS produces a foam height of the order of 15 cm.

Calculations

In order to show how the experimental data have been used, a sample calculation is given in Table 1. The upper section of the table shows how COD and MB values have been employed to calculate the percent disappearance of ABS. The MB values are given in terms of mg/L of COD. It will be noted that the initial values in the Warburg flask for COD and MB are obtained from measurements of the aeration unit effluent, charged with activated sludge to the Warburg run, and the analysis of the ABS feed solution. The contribution in mg/L from the former is $\frac{1}{2}$ the observed value for the aeration effluent because half is discarded when concentrating the sludge prior to the Warburg run, and the remainder (10 ml) is subsequently diluted with 10 ml of the aqueous syndet solution. The results given in the table, then, are on the basis of mg/L for the total volume of solution in the Warburg flask (20 ml).

The calculation of percent metabolism from the oxygen uptakes of the control and fed flasks is shown in the lower section of the table. The observed oxygen uptake values, uncorrected for degree of nitrification, were used. Attempts to provide this type of correction were abandoned when it became apparent that, at the low concentrations involved, the errors in determining nitrogen balances frequently equaled or exceeded the indicated corrections. The calculated percent metabolism incorporates the following assumptions:

1. The difference in oxygen uptake between the fed and control Warburg flasks was assumed to represent oxidation of the syndet. This assumption implies that no significant differences in degree of nitrification existed between the fed and control units. This appears to be reasonable in view of the relatively low concentration of syndet (50 mg/L) and evidence that excess oxygen was available at all times.

2. It was assumed that complete metabolism of the syndet corresponds to 45% of the theoretical oxygen uptake which one would calculate from a knowledge of the empirical formula of the syndet. This value is close to the figure of 40% which has been reported elsewhere (Eckenfelter, 1955).

Table 1. Sample Calculation of Percent Disappearance and Percent Metabolism

Percent Disappearance of ABS

	Chemical Oxygen Demand (COD)	Methylene Blue Extraction (MB)*
Aeration Unit Effluent, mg/L	93	38
Warburg Flask, mg/L:		
Charged with Activated Sludge	47	19
ABS Feed (10 ml)	108	108
Total at Start of Run	155	127
Final Solution, 28 hr	138	104
Amount Disappearing:		
mg/L	17	23
% of ABS Feed	16	21

Percent Metabolism of ABS

Oxygen Uptake after 28 hr, mg/L:	
Control Warburg	25
Fed Warburg	36
Δ', Fed - Control	11
Uptake, % of ABS Feed (108 mg/L)	10
% Metabolism of ABS (10/45)	22

*Absolute values expressed as equivalent mg/L of COD.

Synthesis of Alkylbenzene Sulfonates

In the preparation of the compounds of known structure, the Grignard reaction, followed by dehydration (where required) and hydrogenation, was employed to produce the following alkylbenzenes:

1. 2-Phenyl dodecane from acetophenone and n-decyl bromide.
2. 1-Phenyl-6,8,8-trimethyl nonane from hydrocinnamaldehyde and 1-bromo-3,5,5-trimethyl hexane.
3. 3-Phenyl-6,8,8-trimethyl nonane from propiophenone and 1-bromo-3,5,5-trimethyl hexane.

4. 1,3,3-Trimethyl-5-(3-phenyl propyl) cyclohexane from isophorone and 1-phenyl-3-bromopropane.
5. 1-Methyl-3-(2-phenethyl)-4-isopropyl cyclohexane from menthone and phenethyl bromide.
6. 1-Phenyl-5,5,7,7-tetramethyl octane from bromobenzene and 1-chloro-5,5,7,7-tetramethyl octene-2 (Rohm and Haas).
7. 2-Phenyl-2-methyl undecane from: (a) n-octyl aldehyde and neophyl chloride (the latter prepared from benzene and methallyl chloride using 96% H_2SO_4 at 10 C); (b) n-nonyl bromide and α-bromo cumene (the latter prepared from the benzoyl peroxide — catalyzed reaction of cumene and N-bromo succinimide according to the procedure of Wohl-Ziegler).

The n-alkylbenzenes were obtained as follows:

1. 1-Phenyl-undecane by addition of n-decyl chloride to a sodium dispersion in toluene at 72 C.
2. 1-Phenyl dodecane from Eastman Kodak Company.

In all preparations adequate measures were taken to assure that the final alkylbenzene was free of unsaturated impurities.

The sulfonates were prepared from the alkylbenzenes by reaction with an excess of 20% oleum at 15–50 C over a period of 20 min. After neutralization, the crude sodium sulfonates were recovered, recrystallized twice from water, and dried to yield the final product. This procedure leads to a high concentration of the para sulfonate.

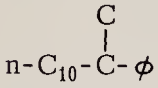
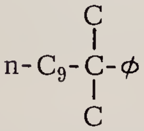
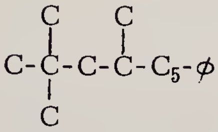
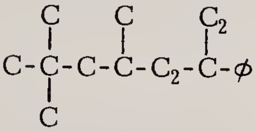
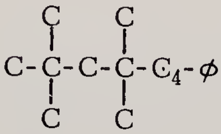
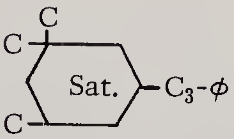
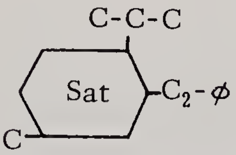
RESULTS

The percent metabolism and percent disappearance of each syndet was calculated for each Warburg run as described in the preceding section. The data obtained in the series of runs (2 to 9 runs) for each syndet were then averaged to give the results assembled in Table 2. Each average has been rounded off to the nearest five units. Average values for the foam height produced from aeration unit effluent samples are also included in the table.

In common with the experiences reported by others, wide fluctuations were observed, on occasion, in the behavior of a given sludge. At best it is difficult to obtain precise results due to the vagaries of living organisms. McGauhey and Klein (1959), as a result of their studies on the biodegradation of alkylbenzene sulfonates, point out the "unpredictable nature of activated sludge in degrading ABS." At the low concentrations employed, analytical errors may also at times limit experimental reproducibility. Thus, the standard errors and confidence limits of the averages for each syndet are rather sizable as shown in the table. However, in spite of these experimental errors, some useful well-defined conclusions have been drawn.

The tabulated data are based on determinations made with due allowance for acclimation of the bacterial sludges to the specific syndet. It is well established that typical bacterial sludges require a length of time to become acclimated to the sulfonate group which does not occur in nature. Preliminary studies also showed that the structure of the alkyl group influences the time required to

Table 2. Biodegradation of Alkylbenzene Sulfonates of Known Structure

Sodium Sulfonate of:	Warburg Unit			Aeration Unit
	% Metabolism*	% Disappearance, Based on:		Foam Produced by Effluent, cm
		COD*	MB*	
1. $n\text{-}C_{11}\text{-}\phi$	90	90	100	0
2. $n\text{-}C_{12}\text{-}\phi$	100	85	100	0
3.  $n\text{-}C_{10}\text{-}\overset{C}{C}\text{-}\phi$	100	85	100	0
4.  $n\text{-}C_9\text{-}\overset{C}{\underset{C}{C}}\text{-}\phi$	85	85	100	0
5.  $C\text{-}\overset{C}{\underset{C}{C}}\text{-}C\text{-}\overset{C}{C}\text{-}C_5\text{-}\phi$	0	35	30	9
6.  $C\text{-}\overset{C}{\underset{C}{C}}\text{-}C\text{-}\overset{C}{C}\text{-}C_2\text{-}\overset{C_2}{C}\text{-}\phi$	<10	†	†	2
7.  $C\text{-}\overset{C}{\underset{C}{C}}\text{-}C\text{-}\overset{C}{\underset{C}{C}}\text{-}C_4\text{-}\phi$	0	65	55	3
8. 	<10	10	30	13
9. 	<10	65	50	1

*Estimated 90% confidence limits:

Compound	Biodegraded	Not Biodegraded
Metabolism	± 15	± 10
COD	± 15	± 10
MB	± 15	± 15

†Unusually large random fluctuations occurred which prevented calculating an average having significance.

achieve acclimation. Acclimation times ranging from 1 to 5 months were provided in the present study in order to minimize the influence of this variable on the results.

DISCUSSION

General Behavior Patterns

The alkylbenzene sulfonates of known structure, which have been studied, fall into two major groups with regard to their behavior in the presence of activated sludge (Table 2). The first four compounds listed are essentially quantitatively metabolized. All of the data are consistent with this conclusion. The 2-phenyl-2-methyl undecane was synthesized by two methods. The two samples behaved in essentially identical fashion.

The remaining compounds resist biodegradation. There is no clear evidence that any significant amount of metabolism has occurred.

In some instances (see compounds 5 and 8) the amounts of syndet removed, as indicated by COD and MB results, are quite low. Large residual proportions of the undegraded syndets thus remain in solution to yield aeration unit effluents which foam severely.

Compounds 7 and 9 give evidence of having disappeared to a sizable extent. Thus, the COD and MB data consistently show appreciable syndet disappearance. At the same time the aeration unit effluents tend to have diminished foaming tendencies in comparison with the effluents mentioned in the preceding paragraph. It appears that adsorption of these compounds by the activated sludge has taken place to a significant degree. In general, the concentration of activated sludge in these two runs was moderately high, i.e., in the range of about 1000 to 1800 mg/L. High solids concentrations would be expected to enhance syndet adsorption. As pointed out below, however, there is evidence that the degree of adsorption of a given syndet may be dependent on its structure as well.

Additional evidence for adsorption lies in the repeated observations (not shown) from the methylene blue test of greater amounts of undecomposed syndet in the *control* Warburg flask at the end of the run than at the start. This indicates that the adsorption is reversible and dependent on the relative concentrations of syndet in the aqueous and sludge phases. For the syndet concentrations used (50 mg/L) and the concentrations of activated sludge present (500 to 1800 mg/L), such adsorption, based on averaged data, is calculated to lie between less than 1 and about 4% by weight of the activated sludge.

Side Chain Structure vs. Biodegradability

Examination of the data in Table 2 from the viewpoint of the effects of side chain structure on the behavior of the alkylbenzene sulfonates leads to a number of interesting observations.

Alkylbenzene sulfonates containing unbranched alkyl groups with the benzene ring at the end of the chain are completely biodegraded under the present experimental conditions. This is shown by the results obtained for the normal undecyl and dodecyl benzene derivatives (rows 1 and 2 of the table). The present results for this type of compound confirm observations previously reported by other investigators (Sawyer, 1958; Sawyer and Ryckman, 1958).

The presence of one or even two methyl branches on the carbon atom adjacent to the benzene ring does not significantly alter the biodegradability of the ABS. This is brought out by comparison of the data for the compounds in rows 2, 3, and 4 of the table. Within the limits of experimental error, all three of these compounds may be regarded as completely metabolized. This result is of particular interest in connection with the compound containing the α quaternary carbon atom. Heretofore, the presence of a quaternary carbon atom in the alkylbenzene sulfonate has been regarded as a basic block to biodegradation. The present data show that this is not the case if the quaternary carbon atom occurs in the closest possible position (α) to the benzene ring, and there is an unbranched, long-chain alkyl group attached to the quaternary carbon.

By way of contrast, a quaternary carbon situated at the far end of the side chain appears to hinder biodegradation strongly. This structure is common to compounds 5, 6, and 7 of the table. In each case the observed degree of metabolism is essentially zero. Since each of the three compounds contains one or more additional branches along the side chain, the observed resistance to biodegradation may not, of course, depend solely on the presence of the quaternary carbon atom. Studies with a compound having a side chain free of such additional branching would be required to establish this unambiguously. Nevertheless, current concepts concerning the mechanism of metabolism lend support to the view that the quaternary carbon atom in this location is a major factor. If, as is generally accepted, biodegradation of a long-chain ABS is initiated at the free terminus of the alkyl group and if metabolism proceeds by the usual process (β-oxidation), the presence of the quaternary carbon atom at this point would clearly be expected to offer resistance to biodegradation.

The presence of a cyclohexyl ring in the side chain hinders biodegradation as shown by compounds 8 and 9 in the table. In addition, the alkyl groups associated with the cyclohexyl ring may influence the adsorption of the compound by activated sludge. The COD and MB data indicate that the compound containing the isopropyl and methyl groups as side chains of the cyclohexyl ring disappears more completely. This difference is confirmed by the foam-producing tendencies of the effluents from the aeration units. The data for both compounds were obtained in the presence of comparable concentrations of activated sludge (1400–1800 mg/L). The difference in behavior of these two compounds supports the view that they possess inherent differences in adsorbability.

SUMMARY

Synthetic detergents are reported to resist biodegradation in sewage-treating plants and receiving waters. The present study was undertaken to give greater understanding of the influence of alkyl group structure on biodegradability of alkylbenzene sulfonates.

Biodegradation studies were conducted using a laboratory activated sludge aeration unit in conjunction with the Warburg respirometer. Measurements were made of percent metabolism and percent disappearance of the sulfonates, as well as the foaming of the aeration unit effluents.

The biodegradability of an ABS detergent is influenced in large measure by the structure of the side chain.

Compounds with strictly linear side chains and those containing one or two methyl branches on the carbon atom attached to the benzene ring are readily biodegradable. The observed biodegradability of the compound containing an α quaternary carbon atom structure is contrary to previously held views. The long, unbranched alkyl group attached to the quaternary carbon apparently provides the means for attack by the microorganisms.

A quaternary carbon atom situated at the free end of the side chain probably hinders biodegradation. Similarly, a compound with a cyclohexyl ring in the side chain strongly resists biodegradation.

Reversible, physical adsorption, dependent on both sludge concentration and side-chain structure, apparently occurs in some instances. It may be a factor in removing resistant structures from solution.

ACKNOWLEDGMENT

The authors acknowledge with sincere thanks the assistance of Dr. C. A. Cohen, who synthesized and made available the sodium sulfonates of 2-phenyl-2-methyl undecane (prepared from n-octyl aldehyde and neophyl chloride) and 1-phenyl-5,5,7,7-tetramethyl octane.

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NONMEDICAL ASPECTS OF ANTIBIOTICS

Philip Gordon, Convenor

Panelists: J. F. Snell
James McGinnis
Thomas G. Pridham

INTRODUCTION

Philip Gordon

Charles Pfizer and Co., Groton, Connecticut

We are slowly beginning to gain some insight into the diverse nature of antibiotic action. No simple answers have appeared, but recent work utilizing the antimetabolite approach, notably that with cycloserine and penicillin, is beginning to bear fruit.

Our contribution to this important antimicrobial area is this symposium: Dr. Snell's paper is a fundamental approach to the mechanism of action of tetracycline antibiotics; Dr. McGinnis correlates the growth responses of antibiotic-fed animals; Dr. Pridham shows the effects of antibiotics, new and old, on the course of plant diseases. These papers represent a distinct advance in our knowledge of antibiotics.

STUDIES ON MODES OF ACTION OF TETRACYCLINES (II)

J. F. Snell and Lorraine Cheng

Radiobiochemistry Department, Charles Pfizer and Co., Maywood, New Jersey

> *To know that we know what we know, and that we do not know what we do not know, that is true knowledge.*
> Confucius

The phrase "mode of action of an antibiotic" conjures up various visions to various people. The content of these visions is somewhat a function of the field of academic training as well as of the individual, but broadly speaking, divides itself according to the various levels of chemical and biological study: the chemist asks what the antibiotic reacts with; the enzymologist asks what enzymes are concerned; the biochemist asks both of these questions, as well as how such a reaction affects the economy of the cell; the bacteriologist and medical doctors ask whether it is bacteriostatic or bactericidal, and against which organisms; and so forth.

It must be admitted that a given antibiotic with many functional groups has many modes of action. This is inevitable, since it also has many modes of reaction with other molecules. This admission may appear to offend certain aspects of the law of parsimony, but it actually does simplify the problem, since it prevents us from asking such unanswerable (and meaningless) questions as "what is *the* mode of action of (for example) tetracycline?" This saves us from searching exclusively for essences such as an absolute or general mode of action (true for every organism and condition) which possibly do not exist. The "mode of action" of tetracycline depends fully as much upon the biological environment in which it is placed as it does upon the nature of the compound itself.

In other words, we probably already know many of the modes of action for the tetracyclines. The problem becomes one of sorting out their various contributions to inhibition and the order in which they apply (as a function of increasing concentration) to various biological systems. Instead of trying to decide between chelation of essential metals, interference with QO_2, inhibition of protein synthesis, etc., as being *the* mode of action, we then adopt the policy of looking into specific biological systems for clues as to the *order* and *conditions* under which one, several, or all of these mechanisms might apply, not excluding, of course, the probability that new "modes of action" will be discovered as information becomes more complete.

We must be as clear as possible about the term "mode of action." This abstraction must be defined with respect to biological level of organization (biological, biochemical, or chemical level), and also with respect to how the over-all mode of action for any particular biological level of organization might vary as a function of increasing concentration of the antibiotic. The following specifications must be made:

1. Biological level of organization of reaction system (cell, enzyme, etc.).
2. *Specific* biological system (from which organism).
3. Conditions of production of 2.
4. Medium in which 2 will be studied.
5. Concentration(s) of antibiotic studied.
6. Parameters measured.

Gale (1952), in an early paper, indicated the diversity of action of a given antibiotic as a function of antibiotic concentration in a study demonstrating that inhibition appeared in four or five reaction systems (protein synthesis, growth inhibition, nucleic acid synthesis, glutamic acid accumulation, and fermentation), at different threshold concentrations, and the inhibition response curve for each reaction system had its characteristic slope: some were inhibited over an extremely narrow range of concentrations in an almost all-or-none fashion (e.g., glutamic acid accumulation from exogenous source), whereas others showed gradually increasing inhibition over a wide range of concentrations (protein synthesis).

These results supply the clue to the conceptual problem of antibiotic "mode of action." Actually, at a given concentration and especially under conditions of antibiotic excess, *modes* of action are probably involved.

It must, however, be granted that for any given biological system, there is probably one reaction which is most sensitive to the antibiotic (Snell *et al.*, 1958; Hahn, 1959); but this is not necessarily the key reaction system identical for every biological system, since this may vary as the relative enzymatic potentialities of biological systems vary. To attempt a more specific statement of this, let us take, for example, the two reactions of protein synthesis and glutamic acid accumulation, as studied by Gale (1952). His information led to the conclusion that protein synthesis was inhibited at ranges of concentration several orders of magnitude lower than his reaction of glutamic acid accumulation. This *might* mean that his experimental conditions for detection of protein inhibition were several orders of magnitude more sensitive than for glutamic acid accumulation (unlikely). The synthetic enzymes for protein synthesis in the organism may be in a region of higher antibiotic concentration than are those responsible for glutamic acid accumulation. Herein would be reflected the importance of the architecture of the cell, the chemical materials out of which it is constructed, as well as its mechanisms for importing and concentrating the antibiotic. Another explanation might maintain that there is no real relationship between the reactions or that they occur at widely different levels in the biochemical economy of the organism, one being essential and the other nonessential.

Now, assuming that all susceptible enzymes were subjected to a given concentration of active antibiotic, one must still contend with variations in the specific affinities of the different susceptible enzymes for the antibiotic. Although a wide range of subtle protective effects are possible (competition with nonspecific adsorptive sites, etc.), one might now expect that the degrees of inhibition of the various enzyme systems would be proportional to the relative stabilities of the enzyme-antibiotic complexes formed, and that the enzyme that catalyzed the most critical reaction (from the standpoint of ability of the cell to reproduce) would represent the point of the primary biochemical lesion. The consequences of this inhibition would then expand out in a chain, involving every reaction which depended upon the primary reaction for substrate, eventually leading to metabolic paralysis. It may not, however, at this point be inferred that this crucial site of inhibition would be identical for every bacterial species whose growth was inhibited by the antibiotic. There may be within the wide range of bacterial types making up the spectrum of the antibiotic more than one such reaction of crucial importance, dependent upon sensitive enzymes in limited supply, elimination of the activity of which might initiate a chain of inhibited reactions leading to bacteriostasis.

The search for these crucial reactions is the proper field for work on modes of action of antibiotics. It is quite likely however that until there is even greater elucidation of basic interrelationships of the various reaction systems in the economy of normal cell metabolism and multiplication, we shall be unable to point with finality to a given reaction system for a given microorganism and say: "This is *the* mode of action of Antibiotic X for Organism A." Meanwhile, we have a multiplicity of leads to follow, which will be summarized below.

Ever since the tetracyclines were discovered about a decade ago, various attempts have been made to elucidate their modes of action. Since the three antibiotics have a common basic structure, and cross resistance among them is usually observed, they were assumed to have similar modes of action (Ciak and Hahn, 1958), although differences do occur in their more detailed modes of action (Snell *et al.*, 1958). For a comprehensive review up to 1955, the reader is referred to Eagle and Saz (1955). Our order of discussion below follows the order of increasing complexity of the chemical or biological reaction system employed for the study.

MODES OF ACTION BASED ON THE COMPLEXING PROPERTIES OF THE TETRACYCLINES

The chemical characteristics of this group of compounds which received the earliest recognition as having something to do with its antimicrobial activities are its chelating and complexing properties (Table 1). Various enzymes have been reported to be inhibited by the tetracyclines due to removal of the essential cations, or to the formation of a toxic drug-metal complex; and certain divalent cations have been reported to reverse this inhibition. Weinberg (1957) covered this aspect in detail in a review in 1957. Thus only the literature that appeared since 1957 is reviewed here.

Interaction with Cations

Price *et al.* (1957) in a series of serial dilution experiments in which the MIC's were determined turbidimetrically, showed that even after an extended period (20 hr) of preincubation of *Pseudomonas aeruginosa* cells with oxytetracycline, exogenous Ca^{++} or Mg^{++} were able to reverse the antibiotic activity. The ratio of cation to antibiotic required was about 50 : 1. This antagonism could then be reversed and antibiotic activity restored by delayed addition of sodium citrate or potassium oxalate. They also found that in the presence of heavy suspensions of washed *Pseudomonas aeruginosa* cells, simultaneous addition of Ca^{++} and oxytetra-

Table 1. Complexing Properties of the Tetracyclines

Complexing Entity	Antibiotic	Reference
Fe^{+++}	OT, CT, T	Albert (1953, 1956)
Al^{+++}	" " "	"
Cu^{++}	" " "	"
Ni^{++}	" " "	"
Fe^{++}	" " "	"
Co^{++}	" " "	"
Zn^{++}	" " "	"
Mn^{++}	" " "	"
Mg^{++}	OT	"
Riboflavin	OT	Huguchi & Bolton (1959)
Desoxyribonucleic acid	OT	"
Adenylic acid	OT	"
Flavin adenine dinucleotide	CT	Yagi *et al.* (1956)

The cations are arranged in the order of decreasing stability of the drug-metal complexes.
OT = oxytetracycline, CT = chlortetracycline, T = tetracycline.

cycline resulted in an unaltered Ca^{++}, but a markedly suppressed oxytetracycline, uptake when compared with the Ca^{++} or oxytetracycline controls. From these data they suggested that the site of antibiotic activity may be at the surface of the cell, and that preferential adsorption of cations was responsible for their antagonism.

Mg^{++} reversal of chlortetracycline and tetracycline inhibition of the respiration of *Azotobacter vinelandii* was observed by Johnson and Colmer (1957). The optimum Mg^{++}-to-antibiotic ratio was 6 : 1 for chlortetracycline, and 1 : 1 for tetracycline. Also, divalent cations were reported to be necessary for chlortetracycline and oxytetracycline inhibition (Rokos, *et al.*, 1958, 1959; Goldman, 1960; Vyshepan and Zueva, 1959). These studies will be discussed again in a later section.

Unpublished studies performed in this laboratory (Snell, unpublished), using radioactive divalent cations, have given evidence that oxytetracycline at low levels increased the uptake of Ca^{45++} or Co^{60++} in or on the cells, whereas higher levels of oxytetracycline effected a decrease. It is possible that at the higher concentrations of oxytetracycline, a more highly chelated metal is formed which penetrates the cells relatively poorly.

Interaction with Compounds of Biological Interest

Foster and Pittillo (1953) found that riboflavin was able to reverse the inhibitory effect of chlortetracycline. They demonstrated that chlortetracycline competitively inhibited the synthesis of riboflavin as well as the utilization of the preformed vitamin, apparently due to a structural analogy between chlortetracycline and riboflavin or its precursor. Even though they did not study oxytetracycline as a competitive inhibitor of riboflavin utilization, they postulated that such a function could very well exist. Yagi *et al.* (1956, 1959) found that chlortetracycline inhibited D-amino acid oxidase by forming a complex with flavin adenine dinucleotide, the binding site being in the riboflavin moiety. Recently Huguchi and Bolton (1959) tested the binding capacities of oxytetracycline with a large number of compounds, including those with biological interest. They found that fused ring compounds of biological interest, such as riboflavin, desoxyribonucleic acid, and adenylic acid exhibited strong binding tendencies with oxytetracycline.

It is also of interest to mention that Stanecki *et al.* (1958) found that culture filtrates from chlortetracycline-resistant *Pseudomonas* and *Proteus* strains could reverse the bacteriostatic action of chlortetracycline on *Staphylococcus aureus*. They concluded that substances which were the products of bacterial metabolism were likely to restore normal cell physiology disturbed by chlortetracycline.

INHIBITION OF SPECIFIC ENZYME SYSTEMS

The tetracyclines have an influence on numerous enzyme systems from microorganisms and mammalian cells (Fig. 1).

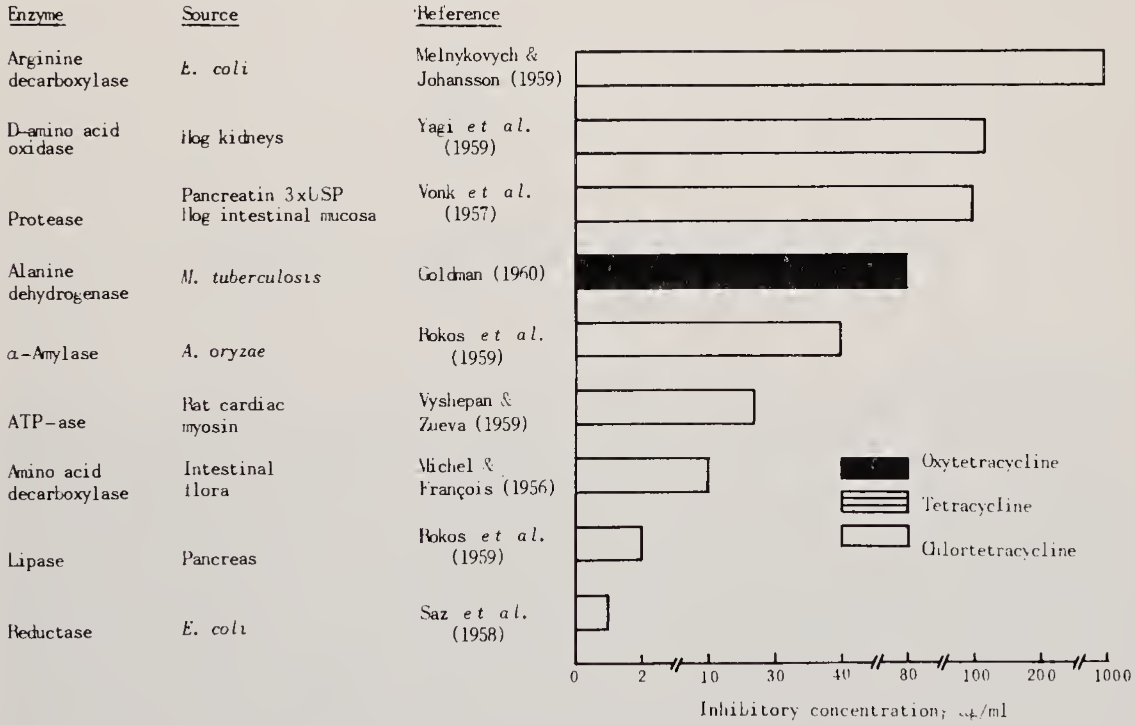

Fig. 1. Inhibition of specific enzyme systems by tetracyclines.

Reductases

Saz and coworkers (Saz and Martinez, 1956; Saz *et al.*, 1956; Saz and Martinez, 1958; Saz and Martinez, 1960) over a period of years, have studied the cell-free reductase from *Escherichia coli* very extensively. They found that cell-free purified extracts from *E. coli* could reduce aromatic nitro compounds to arylamines and could also reduce 2,6-dichlorophenol-indophenol. The preparation which was derived from sensitive cells was sensitive to low levels of chlortetracycline, whereas that from resistant cells was not. The reduction of 2,6-dichlorophenol-indophenol by extracts of sensitive cells was inhibited 36, 59, 80, and 88% in the presence of chlortetracycline concentrations of 0.5, 1.5, 5.0 and 10.0 μg/ml respectively. It must be noted that these levels were growth-inhibiting levels (the organism was inhibited by chlortetracycline concentrations of 1.6-3.2 μg/ml). Hundredfold higher concentrations of tetracycline and oxytetracycline were needed to inhibit the same preparation. This, of course, was a much higher concentration than was needed for growth inhibition. Experimental evidence also showed that resistance correlated with the synthesis of an altered enzyme—one that contained firmly bound conjugated flavin (flavin mononucleotide) in contrast to the easily dissociable flavin of the sensitive preparation. Upon analysis of the flavin content of these extracts, they found that it represented a significant portion of the flavin involved in the electron transport mechanism of the intact cell. Since no reduction of ferricyanide (a single electron acceptor) could be demonstrated, they postulated that chlortetracycline inhibited a two-electron transport mechanism at the stage of reoxidation of the reduced, conjugated flavoproteins. This type of chlortetracycline inhibition can be expressed by the following equations:

$$\text{a) 2,6-dichlorophenol-indophenol} + \text{DPNH} \xrightarrow[\text{aerobic}]{\text{reductase}} \text{reduced 2,6-D} + \text{DPN}$$

$$\text{b) 2,6-dichlorophenol-indophenol} + \text{FMNH} \xrightarrow[\text{anaerobic}]{\text{reductase}} \text{reduced 2,6-D} + \text{FMN}$$

Decarboxylases

The effects of chlortetracycline on bacterial decarboxylases have been studied by several groups of workers. Michel and François (1956) found that chlortetracycline (10 μg/ml) inhibited the decarboxylation of arginine, glutamic acid, aspartic acid, tryptophane, alanine, and methionine by the intestinal flora of the pig. Melnykovych and Johansson (1959) studied the effect of chlortetracycline on *E. coli* arginine decarboxylase, an inducible enzyme which is relatively unstable. They found that chlortetracycline, at 5 μg/ml, protected the enzyme from inactivation (stabilized it), whereas at 1000 μg/ml chlortetracycline accelerated the inactivation of arginine decarboxylase. They maintained that chlortetracycline at low concentration slows down, and at high concentration hastens, the resolution of the coenzyme-apoenzyme complex operating in the decarboxylation of arginine.

Alanine Dehydrogenase

Recently, Goldman (1960) demonstrated that oxytetracycline (80-100 μg/ml) inhibited crude preparations of the alanine dehydrogenase of *Mycobacterium tuberculosis* at high pH values (pH 9-10), but not the purified enzyme. However, when Mg^{++} was added to the purified alanine dehydrogenase preparation, inhibition by oxytetracycline was restored. The other cations which were able to restore the inhibition by oxytetracycline were Fe^{+++}, Fe^{++}, Al^{+++}, Ni^{+++}, Ca^{++}, and Mn^{++}. They concluded that a metal chelate of oxytetracycline was the actual inhibitor, and that in order to permit this chelation and inhibition to take place, the A, B, and C rings of the tetracycline molecule must be intact and the functional groups at C-10, -11, and -12 must be present and unsubstituted.

D-Amino Acid Oxidase

That chlortetracycline inhibited D-amino acid oxidase apparently by forming a complex with flavin adenine dinucleotide (FAD) (Yagi *et al.*, 1956, 1959) has been mentioned in a previous section. This inhibition was found to be due to a competition of chlortetracycline with the oxidase protein for the same binding site on FAD. In the presence of a large excess of substrate and 15 μg of oxidase protein, the inhibition took place at 2.5×10^{-4} M (equivalent to 119.73 μg/ml) chlortetracycline and 1.6×10^{-7} M FAD.

Hydrolases

Rokos and coworkers (Rokos *et al.*, 1958, 1959) have found that chlortetracycline inhibited the activity of α-amylase from *Aspergillus oryzae* and from the pancreas, as well as pancreatic lipase. Chlortetracycline, at a concentration of 8×10^{-4} M inhibited an α-amylase from *A. oryzae*. The inhibitory effect of chlortetracycline was demonstrable only in the presence of Ca^{++}. Thus, in the presence of $0.2\text{-}0.4 \times 10^{-2}$ M $CaCl_2$, inhibition of lipase could be effected by chlortetracycline at a concentration of 50-80 μg/ml. Even at a chlortetracycline concentration of 40 μg/ml, inhibition could take place if sufficient Ca^{++} was present. In the absence of Ca^{++}, chlortetracycline was ineffective at 160 μg/ml. Manganese had a similar effect. In other words, the higher the amount of a given cation in a reaction mixture, the lower the concentration of the antibiotic required for a given degree of inhibition and vice versa. In a later publication, they compared the effects of different divalent metals, and found the order of effectiveness as follows: $Co^{++} > Zn^{++} > Mn^{++} > Ca^{++} > Mg^{++} > Ba^{++}$. Two mM of each cation was used. The lowest chlortetracycline concentration that could bring about a decrease in lipase activity was 2 μg/ml at pH 7-7.9.

Vonk *et al.* (1957) found that chlortetracycline at a level of 5 ppm (equivalent to 5 μg/ml) had no *in vitro* effect on the protease from Pancreatin 3x USP and Intestinal Hog Mucosa, on the amylase from Pancreatin 3x USP, or on cellulase from Rohm and Haas Enzyme 19. These enzymes were typical hydrolases involved in the first stages of digestion. At 100 ppm (equivalent to 100 μg/ml), the antibiotic inhibited protease activity.

ATP-ase

Maxwell and Nickel (1954) found that the ATP-ase activity of rat brain homogenates was not effected by 1×10^{-4} M (about 50 μg/ml) chlortetracycline when incubated in the presence of KCl and $MgSO_4$, in contrast to a stimulatory effect on ATP-ase activity of liver homogenates prepared the same way. However, stimulation of brain ATP-ase was effected when EDTA was present in the rat brain homogenates at a concentration of 1×10^{-4} M.

Interesting to compare with the above study is the finding of Vyshepan and Zueva (1959) who demonstrated that inhibition of ATP-ase from myosin of rat cardiac muscle by chlortetracycline was *only* possible in the presence of Ca^{++}. Thus in the presence of 5×10^{-4} M $CaCl_2$, 24 μg/ml of chlortetracycline could bring about an inhibition. Chlortetracycline did not inactivate the ATP-ase when incubated with KCl.

EFFECT ON OXIDATION

QO_2

The observation that the tetracyclines inhibit oxidation by bacterial cells has been made by various workers (Fig. 2). Some of our data on oxygen uptake by *E. coli* and *S. aureus* in the presence of oxytetracycline is presented in Table 2 (Snell and Cheng, 1958). As low as 1.25 μg/ml of oxytetracycline was able to produce 60% inhibition in *E. coli* 21 (oxytetracycline-sensitive). The same level produced only 4.0% inhibition in *E. coli* 21T (oxytetracycline-resistant). Oxygen uptake in *S. aureus* 209 was also inhibited.

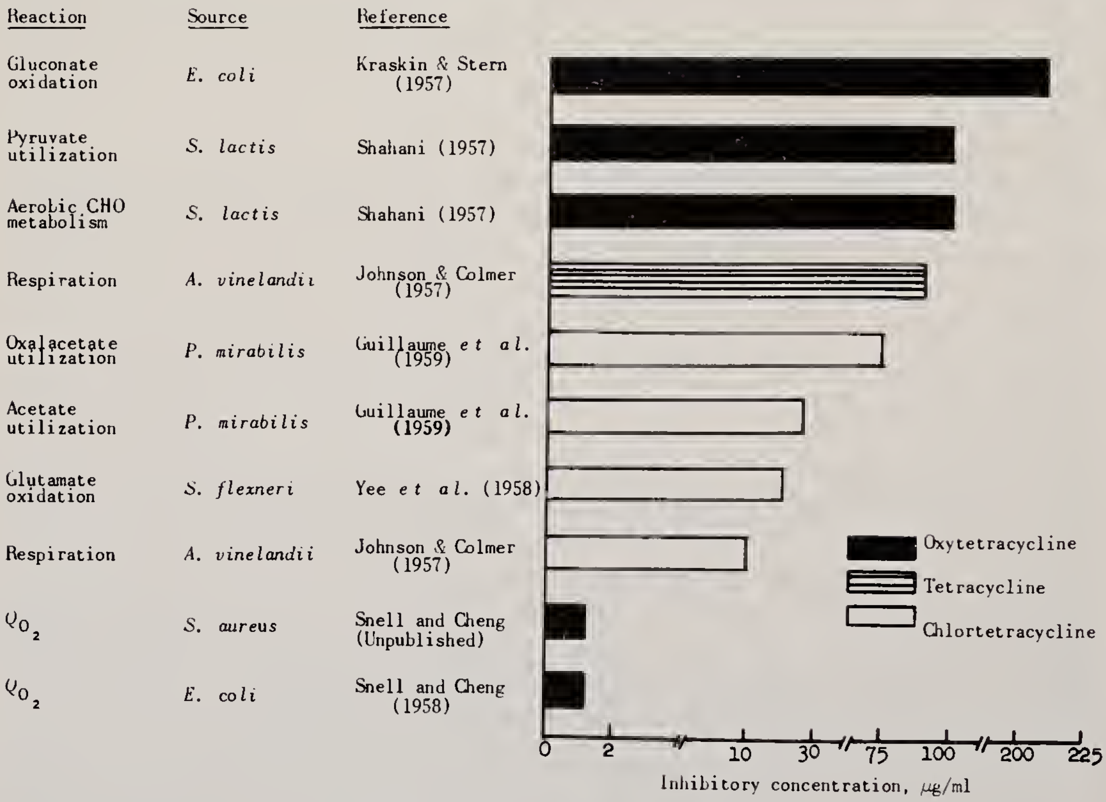

Fig. 2. Inhibition of oxidation by tetracyclines.

In order to relate structural differences in the tetracycline molecule to antibiotic action, we compared a number of derivatives of tetracycline and of glutamic acid (Snell and Cheng, to be published). The effect of the various compounds on the QO_2 of *E. coli* 21 is shown in Table 3. Desdimethylaminooxytetracycline suppressed the QO_2 of *E. coli* 21 to a greater extent than oxytetracycline. 2-Acetyl-2-decarboxyamido oxytetracycline was only slightly inhibitory. Dimethyloxytetracycline was ineffective. D-Glutamic acid and the two glutamic acid derivatives

Table 2. QO_2 of *E. coli* 21 and *S. aureus* 209 at Different Levels of Oxytetracycline HCl

Oxytetracycline Hydrochloride Level μg/ml	QO_2^* (μl O_2/hr/10^8 cells)		
	E. coli 21	*E. coli* 21T[†]	*S. aureus* 209
50	8.7	81.3	199.5
10	9.8	118.9	209.6
2.5	10.1	181.1	207.1
1.25	15.8	197.8	219.9
0	40.0	206.1	384.3

3.5 hours' incubation at 37.5 C in the Warburg apparatus. Oxytetracycline hydrochloride tipped at 30 min. 10×10^8 cells in 2 ml nutrient broth (Difco, pH 6.8) containing 0.05% glucose.

*Calculated over a period of 3.5 hr.

†Oxytetracycline resistant mutant of *E. coli* 21.

Table 3. The Effect of Structurally Related Tetracycline Compounds on the QO_2 of *E. coli* 21

Compounds (0.2 μmoles)	QO_2^* (μl O_2/hr/10^8 cells)
Oxytetracycline HCl	14.8
Anhydrooxytetracycline HCl	15.9
Dimethyloxytetracycline HCl	28.4
Desdimethylaminooxytetracycline[†]	12.0
2-Acetyl-2-decarboxyamido oxytetracycline HCl	22.8
D-Glutamic acid HCl	28.8
N:N-Dimethylglutamic acid HCl	28.8
γ-Methyl-L-glutamic acid HCl	29.7
None	29.5

3.5 hours' incubation at 37.5 C in conventional Warburg apparatus. Compounds tipped at 30 min. 17×10^8 cells in 2 ml nutrient broth (Difco, pH 6.8) containing 0.05% glucose. 12.5 μC/ml sodium acetate-2-C^{14} (specific activity = 2 μC/μmole) added.

*Averaged over the 3-hr period in presence of the compounds.

†A molecular equivalent of hydrochloric acid was added to this compound.

were all without effect. It is of interest to point out that in the case of 2-acetyl-2-decarboxyamido oxytetracycline and dimethyloxytetracycline, where a decrease of inhibitory effect on the QO_2 of *E. coli* was produced, a change in the A ring of the oxytetracycline molecule was involved. The importance of the A ring for bacteriostatic action is strengthened by two recent publications. Goldman's work (1960) has already been mentioned. He found that in order to permit oxytetracycline to chelate with Mg^{++} and thus become inhibitory, the A, B, and C rings of the tetracycline molecule must be intact. Ukita and Arakawa (1957) synthesized a compound having the structure analogous to the A ring of tetracycline. This compound showed antibacterial activity against both *E. coli* and *S. aureus*, though much less effective than tetracycline.

However, the inhibition of oxygen uptake is a rather general phenomenon resulting from more intricate (enzymatic) disturbances in the metabolism of the cell. Several groups of workers have investigated this phenomenon, and many hypotheses have been presented. In order to obtain more details on metabolic changes that accompany QO_2 inhibition, we employed a radioactive precursor, namely, sodium acetate-2-C^{14} (Snell *et al.*, 1958; Snell and Cheng, 1958). By using such an indicator compound, we were not only able to study the oxygen uptake with and without oxytetracycline, but also (by examining the labeled compounds produced from acetate in the broths and cell fractions) investigate the metabolic differences between inhibited and control cultures. These experiments will be discussed in a later section.

Carbohydrate and Pyruvate Metabolism

Shahani and Harper (1958) have observed that the oxytetracycline-resistant strain of *Streptococcus lactis* 10A produced a lower amount of acid when compared with the sensitive strain, even though their growth rates were similar. Since these cultures normally convert sugars to lactic acid by glycolysis with the concomitant formation of sugar phosphate intermediates, they felt that the development of resistance might have been due to an altered phosphorus metabolism. Their study showed that the addition of glucose-1-phosphate and fructose-1,6-diphosphate increased acid production by the resistant cultures, that the P^{32} uptake of these cells was much slower, and that synthesis of certain sugar phosphates was inhibited.

In view of these findings, Shahani (1957) attempted to investigate further the carbohydrate and pyruvate metabolism of these organisms. He found that under both aerobic and anaerobic conditions, the sensitive organisms utilized glucose at a much faster rate than the resistant organisms. The same observation was reported using galactose and lactose as substrates. The resistant organism could not utilize pyruvate at all under either condition, and glucose only aerobically. Oxytetracycline (100 ppm, equivalent to 100 μg/ml) inhibited markedly the aerobic metabolism of carbohydrate and pyruvate utilization by sensitive cells. Since the CO_2 evolution by resistant cells was lower during the anaerobic metabolism of sugars, he postulated that perhaps some nonacidic compound like glycerol or mannitol was produced along with the acid.

Streptococcus lactis normally utilizes sugars to synthesize sugar phosphates and then produces lactic acid via pyruvate. These two studies showed that resistance was accompanied by a loss of phosphorylative ability or a metabolic system (enzymatic and oxidative) connected with the utilization of pyruvate under both anaerobic and aerobic conditions.

At about the same time, Fusillo and Weiss (1957, 1958) published results showing that growth of sensitive and resistant *S. aureus* was the same in a semisynthetic medium. Contrasted to Shahani's findings on *S. lactis*, they found that the ability of the two *S. aureus* cultures to oxidize glucose was also the same. However, the resistant organism manifested an increased oxidation rate of the glucose intermediates (the sugar phosphates), but was unable to oxidize pyruvate. Acetate was not oxidized by either sensitive or resistant cells. They maintained that the resistant organisms could dissimilate carbohydrates under more aerobic conditions than the sensitive organisms, which favored the more anaerobic pathway, and that steps involved in the dissimilation of pyruvate appeared to be a major point of attack. The latter hypothesis is apparently supported by Shahani's finding that oxytetracycline (100 ppm) inhibited pyruvate utilization by sensitive *S. lactis.*

Perhaps the results of Shahani and Fusillo *et al.* can also be interpreted this way: the sensitive cells utilize glucose more readily by anaerobic mechanisms, with the concomitant production of lactic acid through glycolysis, whereas the resistant organisms have stronger oxidative pathways (e.g. for energy purposes).

A similar study was done by Guillaume *et al.* (1959) on *Proteus mirabilis.* They found that chlortetracycline had an inhibitory effect on at least two enzyme systems in the metabolism of glucose and acids of the citric acid cycle. The more sensitive system was that which, in the absence of glucose, permitted the utilization of acetate. This was inhibited by 25 μg/ml of chlortetracycline. The less sensitive system was responsible for the utilization of the dicarboxylic acids, in particular oxalacetic acid. This was inhibited by levels of 75 μg/ml.

Respiration

Johnson and Colmer (1957) found that 2×10^{-5} M chlortetracycline (about 10 μg/ml) could inhibit oxygen uptake (Warburg technique) of *A. vinelandii,* with complete inhibition occurring at 2×10^{-4} M (author claimed lowest level reported). The inhibition caused by 8×10^{-5} M chlortetracycline could be reversed by the addition of Mg^{++}, the optimum molar ratio of Mg^{++} to chlortetracycline being 6 : 1. A concentration of 2×10^{-4} M tetracycline (about 90 μg/ml) could also inhibit respiration, with complete inhibition occurring at 2×10^{-3} M. The Mg^{++} again offered protection, optimum protection occurring at a 1 : 1 molar ratio of Mg^{++} to tetracycline.

Gluconate Oxidation

That oxytetracycline inhibited the oxidation of gluconate was demonstrated by the work of Kraskin and Stern (1957). Evidence was presented which indicated that oxytetracycline competitively inhibited DPN in the electron transport system mediating the oxidation. This reminds one of the study by Saz and coworkers (Saz and Martinez, 1956; Saz *et al.*, 1956; Saz and Martinez, 1958; Saz and Martinez, 1960), in which they postulated that chlortetracycline also interfered with electron transport.

Glutamate Oxidation

That chlortetracycline (20-100 μg/ml) inhibited the oxidation of exogenous glutamate by *Shigella flexneri* was found by Yee *et al.* (1958). Experimental evidence suggested that the drug did not interfere with any of the steps involved in the pathway of glutamate oxidation, i.e., no intermediates appeared to accumulate as a result of the inhibition. The oxidation of glutamate was more sensitive than that of intermediates in the tricarboxylic acid cycle, and the oxidation of glutamate and intermediates of the tricarboxylic acid cycle by cell-free extracts was not inhibited. They postulated that the depression in the rate of glutamate oxidation was due to an interference by chlortetracycline of glutamic acid transport through the cell membrane.

EFFECT ON SYNTHESIS

The tetracyclines have been found to inhibit the formation of a number of adaptive enzymes (Fig. 3).

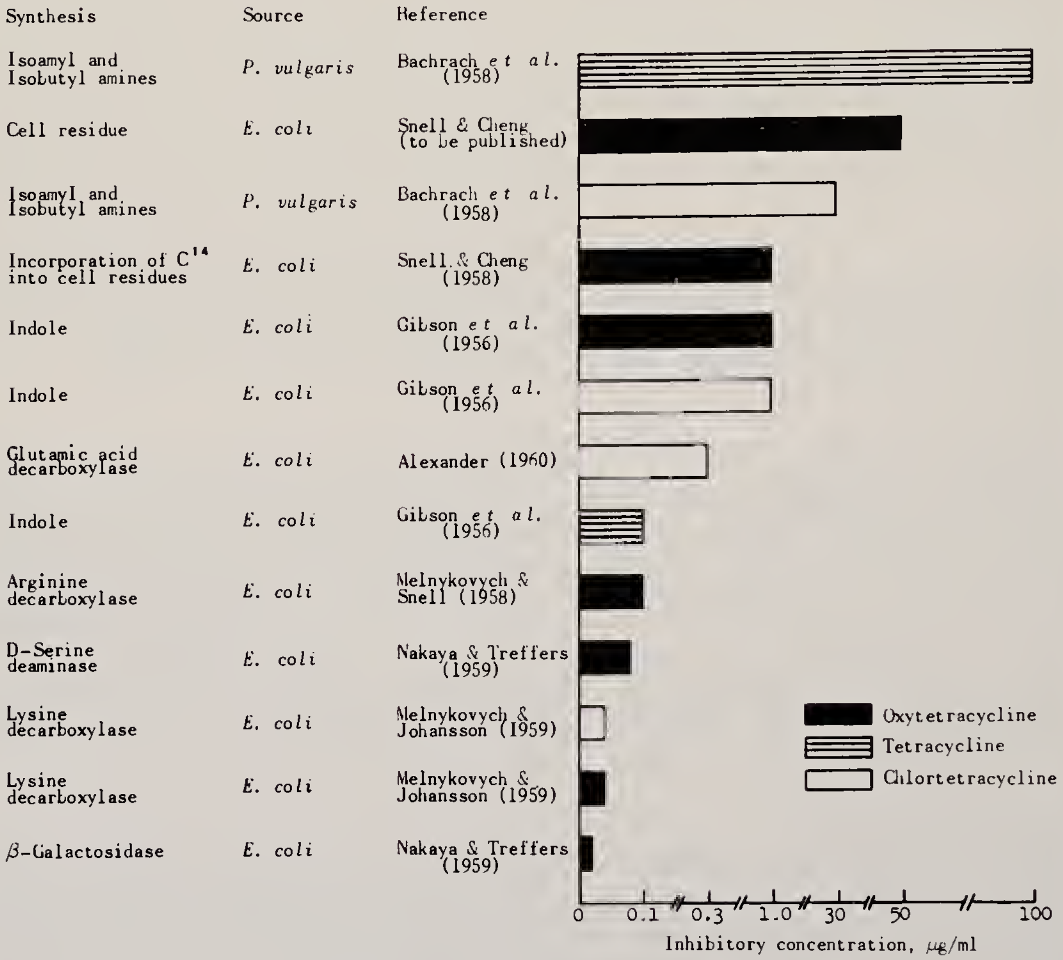

Fig. 3. Inhibition of synthesis by tetracyclines.

Decarboxylases

Melnykovych and Johansson (1959) found that the synthesis of lysine decarboxylase in *E. coli* (Crookes) was inhibited by 0.04 μg/ml of oxytetracycline HCl or chlortetracycline HCl (oxytetracycline being more inhibitory than chlortetracycline). At this concentration, the final pH and turbidity of the culture were not influenced. These antibiotics had a very slight effect on the activity of the preformed lysine decarboxylase. Melnykovych and Snell (1958) found that the formation of arginine decarboxylase by *E. coli* was inhibited by 0.1 μg/ml of oxytetracycline under conditions optimal for the synthesis of this enzyme.

In studying the amino acid decarboxylase activity of *E. coli* isolated from the intestine of chick, Alexander (1960) found that cells grown in the presence of 0.3 μg/ml of chlortetracycline had a lower glutamic acid decarboxylase activity. Further decreases were obtained with 0.5-1.0 μg/ml concentrations. The antibiotic also decreased the uptake of the cofactor for decarboxylation, vitamin B_6.

γ-Amino butyric acid, end product of glutamic acid decarboxylation, was lacking in the culture containing the antibiotic. These findings indicated that chlortetracycline inhibited the formation of glutamic acid decarboxylase by these cultures. The decarboxylase activity was also lower in bacteria from chicks receiving chlortetracycline in the diet. The β-galactosidase activity was greater in cells grown in the presence of chlortetracycline. However, after sonic disruption of the cells, the β-galactosidase activity of cells grown with or without chlortetracycline was equal. The author attributed the difference in β-galactosidase activity of the whole cells to the effect of chlortetracycline on cell permeability.

Amines

Related to the inhibition by the tetracyclines of amino acid decarboxylase synthesis is the effect of tetracyclines on the formation of amines by bacteria. Bachrach *et al.* (1958) studied this effect using *Proteus vulgaris* and *Streptococcus faecalis* isolated from human feces. The formation of isoamyl amine and isobutyl amine by *P. vulgaris* was inhibited by 30 μg/ml of chlortetracycline or 100 μg/ml of tetracycline. These levels showed no inhibition of growth at the time interval when inhibition of amine formation was already apparent. The formation of the aromatic amines, tyramine and phenylethyl amine by *S. faecalis* was not inhibited by subinhibitory (for growth) concentrations of tetracyclines but was inhibited by higher concentrations.

β-Galactosidase and D-Serine Deaminase

The induction of β-galactosidase and D-serine deaminase in *E. coli* K12 was found by Nakaya and Treffers (1959) to be inhibited by oxytetracycline and chloramphenicol. Thus 0.018 μg/ml of oxytetracycline inhibited β-galactosidase activity, and 0.08 μg/ml inhibited the activity of D-serine deaminase.

Indole

Gibson *et al.* (1956) found that indole synthesis from glucose, NH_4Cl, and serine by washed suspensions of *E. coli* 7-4 was inhibited by bacteriostatic concentrations of chloramphenicol and the tetracyclines (1 μg/ml). Even at lower levels, at which growth was not inhibited, a detectable inhibition of indole synthesis took place. Thus at 0.1 μg/ml, tetracycline inhibited indole synthesis but did not inhibit growth. The inhibition did not appear to be on adaptive enzyme formation, because the tetracyclines still inhibited indole synthesis when added to suspensions which were forming indole at maximum rate. Indole synthesis by suspensions of antibiotic-resistant variants of *E. coli* 7-4 was not inhibited by concentrations of antibiotics which inhibited synthesis by the parent strain.

Glutamic Acid

Our approach to mode of action has been through the radioprofile technique (Snell *et al.*, 1958), i.e., the inhibited and uninhibited organisms are given the same radioactive indicator to metabolize, and the ways in which they metabolize it are compared. The differences in the distribution of radioactivity in the various cell fractions (broth, trichloroacetic acid extracts, cytoplasmic protein, cell membrane, cell wall, etc.), and the undue accumulation of a particular metabolite in any one of the fractions is used then as a clue to the possible site of antibiotic action under the experimental conditions employed.

We have studied the effects of the tetracycline antibiotics on the metabolism of 2-C^{14} acetate by sensitive and resistant strains of *E. coli*. By procedures which have been described in detail (Snell *et al.* 1958) the characteristic quantitative differences in broths were obtained by paper chromatography and radioscanning, producing tabulated data as shown in Fig. 4. The results of early experiments in this manner produced a table (Table 4) of unknown spots from the oxytetracycline-sensitive strain which were absent from, or quantitatively negligible in, the sensitive control broths; and absent from the resistant culture broths, both in the presence and absence of antibiotic.

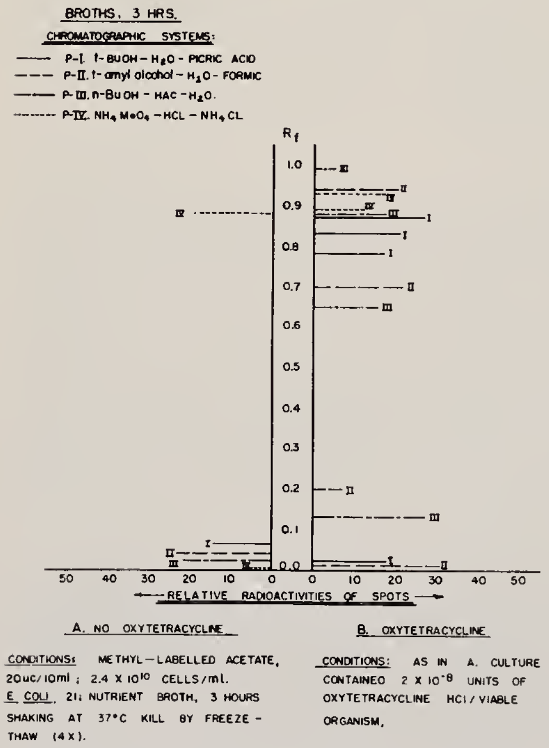

Fig. 4. Radioprofile of *E. coli* 21 on methyl-labeled acetate in the presence and absence of oxytetracycline.

Later (Snell and Cheng, 1958), one of the compounds (*E. coli* 21α) accumulated in oxytetracycline-inhibited *E. coli* fermentation broth was found to be glutamic acid. This glutamic acid was endogenously synthesized from the radioactive precursor, sodium acetate-2-C^{14}. At 1.25 μg/ml of oxytetracycline, 9.7% of the original radioactivity was incorporated into glutamic acid by *E. coli* 21, whereas in the absence of antibiotic, it incorporated only 1.7% (Table 5). An accumulation of several other compounds labeled by the 2-C^{14}-acetate precursor was also found, but none as highly labeled as glutamic acid, and none of them increased proportionately with increasing oxytetracycline levels (1.25-50 μg/ml) as did glutamic acid. Furthermore, none were amino acids.

Further examination of the accumulated C^{14}-glutamic acid, by means of a combination of enzymatic and microbiological techniques, revealed that, in the presence of 50 μg/ml of oxytetracycline, it was all of the D-configuration (Snell and Cheng, 1959). Since D-glutamic acid was known to be a component of the bacterial cell wall (Ikawa and Snell, 1956; Strange and Thorne, 1957), the question arises as to whether oxytetracycline interferes with the normal course of incorporation of D-glutamic acid into the bacterial cell wall.

The accumulation of C^{14}-glutamic acid from acetate precursor in the presence of antibiotics has not to our knowledge been previously reported. Its concurrent absence from resistant *E. coli* broths was also striking. Our interest in glutamic acid was intense at the time of these studies because of parallel research we were doing on the mode of biosynthesis of radioactive oxytetracycline from 2-C^{14} acetate (Snell *et al.*, 1956), a biosynthesis which we reported in 1955. Figure 5 shows the structural relationship we have postulated (based on specific activity of degradation products, etc.) between the A ring of the tetracycline antibiotics

Table 4. Oxytetracycline-induced C^{14}-Labeled Spots in *E. coli* 21 Fermentation Broth

Spot	R_f in System	
	P-II *	P-III †
EC 21 - α	0.366 ± .021	0.317 ± .026
EC 21 - β	0.533 ± .030	0.472 ± .015
EC 21 - γ	0.599 ± .030	0.530 ± .010
EC 21 - δ	0.722 ± .024	0.718 ± .018
EC 21 - ϵ	0.856 ± .017	0.856 ± .022
EC 21 - ζ	0.948 ± .008	

3.5 hours' incubation at 37.5 C. Oxytetracycline HCl (1.25-50 μg/ml) tipped at 30 min. 10×10^8 cells in 2 ml nutrient broth (Difco, pH 6.8) containing 0.05% glucose. 6 μC/ml NaAc-2-C^{14} added. Contents of duplicate flasks were pooled for radioassay.

*Tertiary amyl alcohol, distilled water, formic acid, 3 : 3 : 1.

†Normal butanol, glacial acetic acid, distilled water, 74 : 19 : 50.

Table 5. Glutamic Acid Radioactivity in *E. coli* 21 Fermentation Broths

Oxytetracycline Hydrochloride Level μg/ml	Glutamic Acid	
	Per cent of Total Radioactivity (24 μC)	Per cent of Broth Radioactivity
50	21	37
10	20.5	32
2.5	12.5	25
1.25	9.7	13
0	1.7	5.3

3.5 hours' incubation at 37.5 C in the Warburg apparatus. Oxytetracycline hydrochloride tipped at 30 min. 10×10^8 cells in 2 ml nutrient broth (Difco, pH 6.8) containing 0.05% glucose. Six μC/ml NaAc-2-C^{14} added. Contents of duplicate flasks were pooled for radioassay. Chromatography in System P-II (tertiary amyl alcohol, distilled water, formic acid, 3 : 3 : 1).

and glutamic acid (Snell *et al.*, 1960). We were, hence, interested in the possible relationship of the A moiety of the oxytetracycline molecule to interferences with the metabolism of glutamic acid in *E. coli*. One might consider oxytetracycline to act as an analogue of glutamic acid, and thus to compete with glutamic acid for binding site(s) on the same enzyme system (either apoenzyme or coenzyme). If such were the case, then it would be logical to suppose the A ring of the tetracycline molecule to be an important moiety for its antibiotic activity (see page 115).

In connection with the influence of substituent groups on the activity of the tetracycline structure, the paper of Beereboom *et al.* (1960) is of great interest. It would appear that the substitution of the molecule in the 9 position with a nitro group has a large influence on the specific antibiotic bioactivity against *Klebsiella pneumoniae.* However, as pointed out by the authors, the same relationships do not necessarily hold for other microorganisms. This loss in potency, then, might be a reflection of a modification in the ability of the antibiotic to penetrate (in *K. pneumoniae*) to the site of action in sufficient concentration to produce inhibition, rather than in a modification of the portion of the molecule mainly responsible for inhibition.

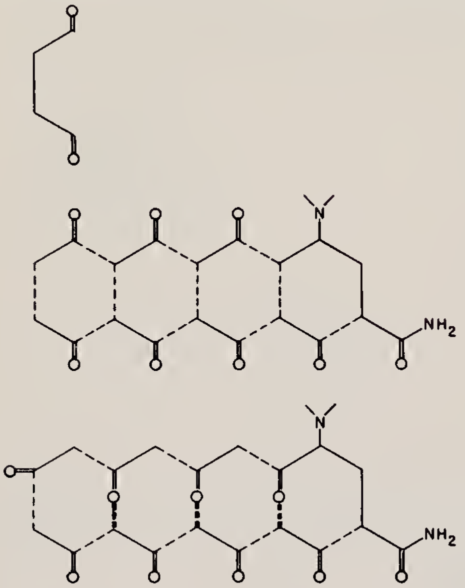

Fig. 5. Suggested biosynthesis of tetracycline nucleus.

The accumulation of D-glutamic acid in *E. coli* 21 (oxytetracycline-sensitive) implies a cessation of cell wall synthesis accompanied by an accumulation of cell wall precursor compounds. A question of interest would be whether the specific activity of the glutamic acid accumulating from acetate in the blocked culture is similar to that appearing in the cell wall in the unblocked culture; whether exogenous glutamate affected the relationship between the specific activities of cell wall and broth glutamate; and whether the rate of accumulation of this D-amino acid is in agreement with the rate at which it might be expected to be utilized for cell wall synthesis in the unblocked cells (in this connection, see Park and Strominger, 1957). These studies are incomplete at this time, but studies have been initiated in which we investigated the incorporation of C^{14}-radioactivity from the sodium acetate-2-C^{14} precursor into the glutamic acid of different cell fractions of *E. coli.* Several tetracycline antibiotics and some of their derivatives, as well as glutamic acid and some of its derivatives, were employed, and their effects on this incorporation as well as activities of the glutamic acid fractions were studied. The details of these studies are to be published elsewhere. Some data will be presented here.

Table 6 shows the relative percentages of total and broth radioactivity accounted for by the accumulated glutamic acid. It is interesting to note that glutamic acid accumulation in the broth was a common phenomenon for the control cells and antibiotic-inhibited cells up to 1 hr; subsequently, glutamic acid continued to accumulate in the broth of the inhibited cells, whereas that in the broth of the control cells began to disappear. During the first ½-hr of contact, the rate of accumulation was most rapid in the presence of oxytetracycline.

At this point, it was reasonable to consider the consequences of assuming that interference with glutamic acid metabolism of oxytetracycline might take place in a directly competitive way due to the structural similarities pointed out. We have therefore performed preliminary experiments designed to determine

Table 6. Glutamic Acid Radioactivity in *E. coli* 21 Fermentation Broths

Compounds (0.2 μmoles)	% Glutamic Acid Over Different Time Intervals					
	1/2 Hr		1 Hr		3 Hr	
	% Added Radio-activity (25 μC)	% Broth Radio-activity	% Added Radio-activity (25 μC)	% Broth Radio-activity	% Added Radio-activity (25 μC)	% Broth Radio-activity
Oxytetracycline HCl	2.1	18.1	2.8	16.5	11.4	18.0
Chlortetracycline HCl	1.2	10.0	1.8	13.2	18.5	22.4
Tetracycline HCl	0.2	2.6	4.2	22.6	12.5	24.8
Desdimethylaminooxytetracycline*	0.1	0.8	0.8	7.1	–	–
Terracinoic Acid†	–	–	–	–	0.0	0.0
Chloramphenicol	0.1	0.8	3.9	20.3	18.1	25.3
None	1.1	8.9	3.6	27.3	0.0	0.0

Incubation at 37.5 C in the Warburg apparatus. 10×10^8 cells in 2 ml of nutrient broth (Difco, pH 6.8) containing 0.05% glucose. 12.5 μC/ml NaAc-2-C^{14} added.

*Desdimethylaminooxytetracycline was studied at 1/2- and 1-hr intervals only.

†Terracinoic acid was studied at the 3-hr interval only.

whether the addition of cold glutamic acid to oxytetracycline-inhibited *E. coli* (50 μg/ml) affected in any way the synthesis and accumulation of radioactive glutamic acid from 2-C^{14} acetate in the broth. The added glutamic acid might affect the amount of C^{14}-glutamic acid appearing in the broth by any of the following mechanisms or combinations thereof: 1) direct interference, in a competitive way with oxytetracycline on synthesis or utilization of glutamic acid; 2) concentration intracellularly in sufficient quantities to increase (by displacement) or decrease the excretion of hot glutamate; 3) use as a substrate for further oxidation intra- or extra-cellularly. Table 7 illustrates the effects produced by

Table 7. Effect of Exogenous Glutamic Acid on Metabolism of *E. coli*

Level of Glutamic Acid* (μmoles/ml)	Radioactivity from 2-C^{14} Acetic Acid, % of Total						
	Broth	KOH	Freeze-thaw	Cold TCA	75% EtOH	Hot TCA	Residue (by difference)
0.00	12.4	33.6	5.72	5.92	5.0	4.0	33.36
0.10	57.9	12.0	2.72	1.40	3.0	1.32	21.66
1.00	64.0	7.92	2.40	1.40	3.40	0.92	19.96
10.00	58.8	8.40	2.40	1.32	3.72	0.92	24.44
100.00	52.0	2.64	2.20	1.20	2.92	0.60	38.44

Conditions same as for Table 5.

*All flasks contained 0.1 μmole oxytetracycline HCl per ml.

the addition of graded amounts of glutamic acid under conditions of oxytetracycline inhibition. Bearing in mind the previously shown data on graded oxytetracycline inhibition, it is seen that, at the constant maximal level of oxytetracycline inhibition used here, the addition of high levels of glutamic acid elicited effects on radioactive glutamic acid accumulation similar to those produced by graded levels of oxytetracycline alone. For example, in the presence of small amounts of added glutamic acid (0.1 μmole/ml) the distribution picture looks very much like that produced by 2.50 μg/ml oxytetracycline alone. Radioactive glutamic acid in the medium increases as the concentration of cold DL-glutamic acid added to the medium increases. It appears that the added glutamic acid is effecting the release of C^{14}-glutamate from the cells.

Table 8. Distribution of Radioactivity in Different Fractions of *E. coli* 21

Compounds (0.2 μmoles)	% of Total Added Radioactivity (25 μC) in Fractions								Total Recovery %
	KOH	Broth	Aqueous	Cold TCA	Hot TCA	Acidified 75% EtOH	Ether	Hydrolyzed Cell Residue	
Oxytetracycline HCl	19.4	84.0	3.6	2.6	2.7	6.0	<0.1	2.1	120.40
Anhydrooxytetracycline HCl	27.8	57.2	6.7	2.0	4.4	8.0	0.29	4.1	110.49
Dimethyloxytetracycline HCl	29.5	14.0	6.2	6.2	7.1	17.0	0.35	14.0	94.35
Desdimethylaminooxytetracycline	12.2	77.6	4.6	0.6	1.4	7.0	0.14	2.4	105.94
2-Acetyl-2-decarboxyamido oxytetracycline HCl	31.7	41.2	14.5	1.4	6.4	11.5	0.35	5.0	112.55
D-Glutamic acid HCl	35.5	10.4	5.7	8.0	7.4	21.5	0.50	21.0	110.00
N:N-Dimethylglutamic acid HCl	36.0	11.6	5.4	9.0	7.6	15.8	0.36	16.6	102.36
γ-Methyl-L-glutamic acid HCl	36.9	17.2	5.4	9.7	7.3	12.2	0.26	16.0	104.96
None	35.1	10.0	5.3	8.8	8.0	14.5	0.43	18.7	100.83

Conditions same as for Table 3.

Table 8 shows how the different structurally related compounds affected the distribution of radioactivity in the various fractions. The radioactivity in the KOH fraction corresponded with the QO_2 data (see Table 3), since it represented the respiratory $C^{14}O_2$. The lowest level of KOH radioactivity occurred in the presence of desdimethylaminooxytetracycline, being 35% of the control. The QO_2 in this case was 41% of the control.

Although desdimethylaminooxytetracycline was most effective in inhibiting the respiration of *E. coli* 21, the highest retention of radioactivity in the broth occurred in the presence of oxytetracycline (an increase of more than eightfold over the control). Desdimethylaminooxytetracycline, anhydrooxytetracycline, and 2-acetyl-2-decarboxyamido oxytetracycline followed in decreasing order (eight-, six-, and fourfold increases respectively). No significant increase was produced by dimethyloxytetracycline or the glutamic acid derivatives. A high retention of radioactivity in the broth was accompanied by a low incorporation of radioactivity into the cell extracts.

The final cell residue must contain a significant amount of cell wall and membrane material (Snell *et al.*, 1955; Park and Hancock, 1960). The data indicate a significantly lower level of radioactivity in the hydrolyzed cell residues from oxytetracycline- and desdimethylaminooxytetracycline-containing cultures.

Table 9. Distribution of *de novo* C^{14}-Glutamic Acid in *E. coli* 21

	C^{14}-Glutamic Acid in Fractions						Total C^{14}-Glutamic Acid*	
	Broth		Aqueous		Hydrolyzed Cell Residue			
Compounds (0.2 μmoles)	μC	% of Total Glutamic Acid Activity	μC	% of Total Glutamic Acid Activity	μC	% of Total Glutamic Acid Activity	μC	%
Oxytetracycline HCl	3.08	86.5	0	0	0.48	13.5	3.56	100
Anhydrooxytetracycline HCl	0.80	58.5	0	0	0.57	41.5	1.37	100
Dimethyloxytetracycline HCl	0.13	8.0	0	0	1.50	92.0	1.63	100
Desdimethylaminooxytetracycline	0.70	65.0	0	0	0.38	35.0	1.08	100
2-Acetyl-2-decarboxyamido oxytetracycline HCl	0.21	25.6	< 0.03	< 3.7	0.58	70.7	< 0.82	100
D-Glutamic acid HCl	< 0.05	< 1.8	0.08	2.9	2.62	95.3	< 2.75	100
N:N-Dimethyl glutamic acid HCl	0.08	4.8	0.06	3.6	1.53	91.6	1.67	100
γ-Methyl-L-glutamic acid HCl	0.08	2.1	1.50	40.2	2.15	57.7	3.73	100
None	0	0	1.25	33.0	2.55	67.0	3.80	100

Conditions same as for Table 3.
*Calculated by totalling the glutamic acid radioactivity from each of the three fractions.

Chromatography of each of the culture fractions indicated that glutamic acid was only present in the broth, the aqueous cell extract, and the hydrolyzed cell residue. Table 9 shows the levels of C^{14}-glutamic acid in each of these fractions. Since the level is expressed in terms of total radioactivity, it represents the glutamic acid synthesized *de novo* from the C^{14}-acetate precursor. Assuming the specific activities of the synthesized glutamic acids were similar, the total *de novo* synthesis in the oxytetracycline-inhibited cells and in the control cells was the same. The difference lay in the distribution of this glutamic acid. More than 87% of the total glutamic acid was in the broth of the oxytetracycline-treated cells, while only 13% was incorporated into the cell residue. In the control cells, no glutamic acid could be found in the broth, while 67% of the total glutamic acid was in the hydrolyzed cell residue. The remainder was in the aqueous extract. These results indicate an alteration in cell permeability, possibly due to damage in the cell wall and/or membrane. Alexander (1960) also showed that chlortetracycline affected the permeability of *E. coli*.

Table 10 shows that more than 90% of the radioactivity in the hydrolyzed cell residue from oxytetracycline-inhibited culture was in glutamic acid. In the desdimethylaminooxytetracycline-treated cell residue, glutamic acid only comprised 64% of the radioactivity. In the case of the other compounds, glutamic acid radioactivity fell to 40-55%. On the basis of dry weight of cell residue and % of total radioactivity, the incorporation of C^{14}-glutamic acid was lower for all of the antibiotic-treated cells.

Table 10. Incorporation of C^{14}-Glutamic Acid into Cell Residues of *E. coli* 21

Compounds (0.2 μmoles)	C^{14}-Glutamic Acid		
	% of Cell Residue Activity	μC/mg, Dry Weight of Hydrolyzate	% of Total Activity (25 μC)
Oxytetracycline HCl	92.4	0.654	1.90
Anhydrooxytetracycline HCl	55.0	0.564	2.26
Dimethyloxytetracycline HCl	42.9	0.606	6.00
Desdimethylaminooxytetracycline	64.1	0.411	1.53
2-Acetyl-2-decarboxyamido oxytetracycline HCl	45.6	0.445	2.34
D-Glutamic acid HCl	45.7	1.414	9.60
N:N-Dimethyl glutamic acid HCl	36.8	0.715	6.13
γ-Methyl-L-glutamic acid HCl	53.0	0.958	8.47
None	54.7	1.491	10.20

Conditions same as for Table 3.

The nitrogen contents and dry weights of the hydrolyzed cell residues are given in Table 11. The values for the oxytetracycline-inhibited cells were much lower than those for the control cells.

Gale and Folkes (1953b) found that at bacteriostatic concentrations of chlortetracycline and oxytetracycline, protein synthesis was almost completely inhibited, while the inhibition of free glutamic acid accumulation (from an exogenous pool) took place at a higher concentration. Presumably a cell wall or membrane was necessary for the transport of glutamic acid into the cell's internal environment. Rowlands *et al.* (1957) later demonstrated that the presence of a "barrier" was indeed necessary. Since a source of energy was required for the accumulation, a process of active transport involving "permeases" (Cohen and Rickenberg, 1956) was suggested. Thus, what Gale and Folkes observed as an inhibition of protein synthesis could mean also an inhibition of the synthesis of enzymes such as permeases in the cell membrane, which eventually led to an inhibition of free glutamic acid accumulation within the cell. The significance of the accumulation reaction per se for cell growth remains obscure.

Gale and Folkes (1953a) used the glutamate content of the hydrolyzate of the cold trichloracetic acid – precipitable material from cell suspensions as a measure of the protein present. This material must necessarily contain the cell walls and membranes. Therefore, what they estimated as "combined glutamate" did not differentiate between the glutamic acid incorporated into the cell wall and membrane fraction and that into the cytoplasmic protein fraction. Thus the measure of "combined glutamate" does not indicate protein synthesis in the strict sense.

Table 11. Nitrogen Contents and Dry Weights of Hydrolyzed Cell Residues

Compounds (0.2 μmoles)	Hydrolyzed Cell Residues		
	Nitrogen (μg)	Dry Weight (μg)	Nitrogen % of Dry Wt.
Oxytetracycline HCl	52.5	725	7.25
Anhydrooxytetracycline HCl	62.5	1,005	6.22
Dimethyloxytetracycline HCl	190.0	2,465	7.71
Desdimethylaminooxytetracycline	73.8	920	8.02
2-Acetyl-2-decarboxyamido oxytetracycline HCl	85.0	1,323	5.15
D-Glutamic acid HCl	188.0	1,820	10.33
N:N-Dimethylglutamic acid HCl	192.5	2,140	9.00
γ-Methyl-L-glutamic acid HCl	212.5	2,210	9.61
None	227.5	1,710	13.30

Conditions same as for Table 3.

In the present experiment, lower values for C^{14}-glutamic acid, total dry weight, and total nitrogen content were obtained from the residues of oxytetracycline-inhibited cells, in comparison with those obtained from control cells. Assuming that cell wall and membrane material must occupy a significant portion of the cell residue (Snell *et al.*, 1955; Park and Hancock, 1960), these data, together with those reported previously (Snell and Cheng, 1958 and 1959), seem to point toward a preferential inhibition of the synthesis of cell wall and/or membrane material rather than inhibition of protein synthesis in general.

In view of recent findings, protein synthesis and cell wall and/or membrane synthesis seem to be two closely related processes. Butler *et al.* (1958) and Brookes *et al.* (1959) showed that protein synthesis depended on the cell membrane. They demonstrated that the site of protein synthesis in *Bacillus megaterium* was probably located on or closely associated with the cytoplasmic membrane. This indicates that damage in these structures would eventually lead to inhibition of protein synthesis.

CONCLUSION

What are we to conclude from this large compendium of reactions, at various chemical and biological levels, about the mode of action of tetracyclines? It seems that there are few conclusions, but many more questions to answer. In surveying the efforts that have been made, it is important to distinguish between those who have studied their effects at a limited number of antibiotic concentrations, and those who have titrated the antibiotic down to a level of concentration at which no effect on their reaction system was apparent. The great bulk of the effects summarized in Figs. 1 to 3 are simply those that were obtained at the lowest concentration of antibiotic tried. How significant the effect might be at still lower concentrations was not, in general, reported. This means that we do not have available sufficient data to construct a true picture of the *relative sensitivities* of the various reactions systems reported to be inhibited. The best that we can do in this regard, is to place what data *was* reported in the order of increasing concentrations. On this basis, Figs. 6 and 7 have been prepared.

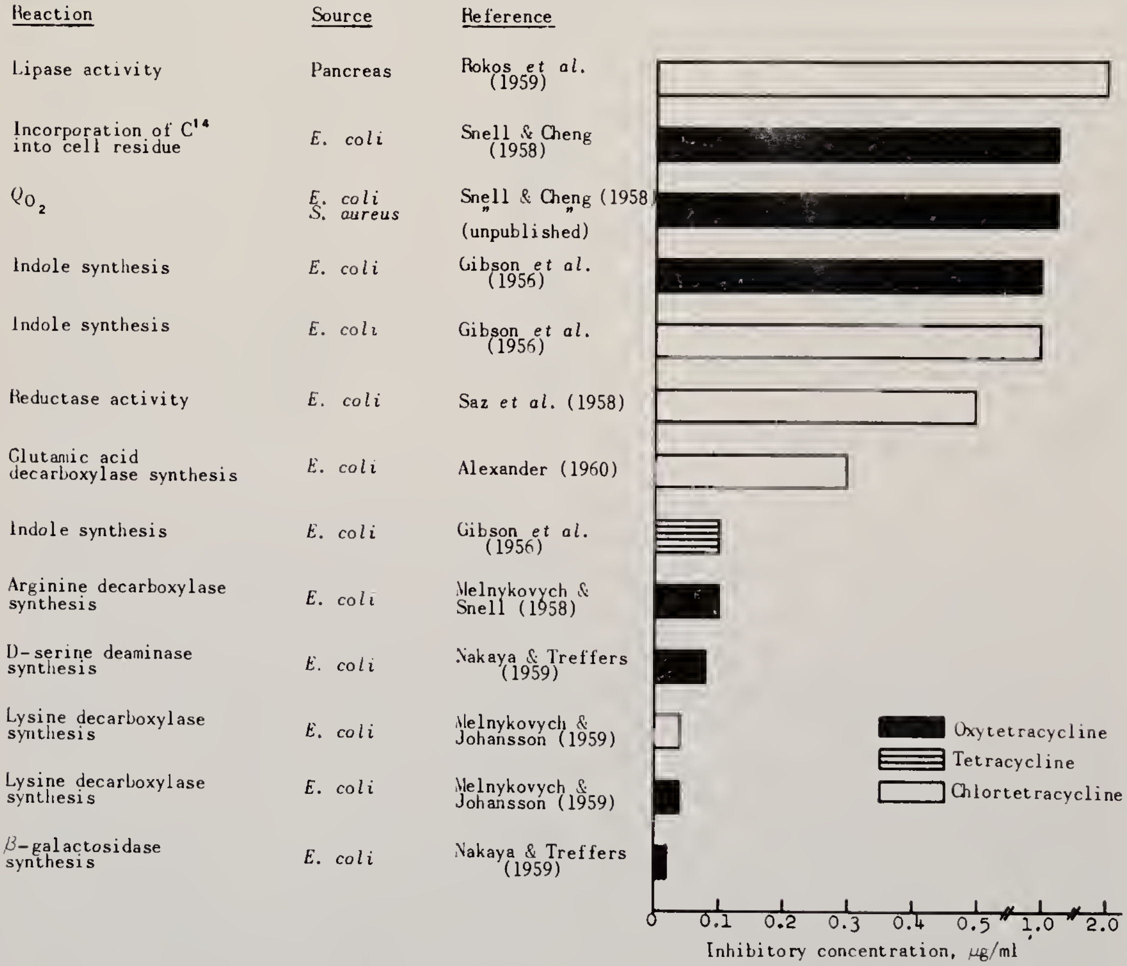

Fig. 6. Inhibition by tetracyclines (0–2 μg/ml).

From this arrangement of the data, it would appear that the synthetic reactions in *E. coli* for *β*-galactosidase, lysine decarboxylase, D-serine deaminases, arginine decarboxylase, and indole have been titrated down to the lowest concentrations of antibiotic with inhibitory effects at less than 0.1 μg/ml; among the oxidative reactions of the cell, the Q_{O_2} of *E. coli* and *S. aureus* are markedly inhibited at about 1 μg/ml, and nitro reductase is inhibited at about 0.5 μg/ml. If one wished to base his ideas of mode of action on these reactions, which have been demonstrated to occur in the same range as growth inhibition, the following objection might be anticipated: We know so little about the relative criticality of the above reactions to the total economy of a given microorganism that we are not in a position to say that blocking any one of them would necessarily deal the *coup de grâce* to the microorganism. Another way of stating this is to say that, so far as we know, there does not seem to be one reaction among them which is *known* to be essential enough under varied conditions of growth and within several species of organism to serve as a locus of the primary biochemical lesion produced by the antibiotic at the level that would account for the growth inhibition observed.

Reaction	Source	Reference
Gluconate oxidation	*E. coli*	Kraskin & Stern (1957)
D-amino acid oxidase activity	Hog kidneys	Yagi *et al.* (1959)
Amine synthesis	*P. vulgaris*	Bachrach *et al.* (1958)
Pyruvate utilization Aerobic CHO metabolism	*S. lactis*	Shahani (1957)
Respiration	*A. vinelandii*	Johnson & Colmer (1957)
Alanine dehydrogenase activity	*M. tuberculosis*	Goldman (1960)
Oxalacetate utilization	*P. mirabilis*	Guillaume *et al.* (1959)
Cell residue synthesis	*E. coli*	Snell & Cheng (to be published)
α-amylase activity	*A. oryzae*	Rokos *et al.* (1959)
Amine synthesis	*P. vulgaris*	Bachrach *et al.* (1958)
Acetate utilization	*P. mirabilis*	Guillaume *et al.* (1959)
ATP-ase activity	Rat cardiac myosin	Vyshepan & Zueva (1959)
Glutamate oxidation	*S. flexneri*	Yee *et al.* (1958)
Respiration	*A. vinelandii*	Johnson & Colmer (1957)
Amino acid decarboxylase activity	Intestinal flora	Michel & François (1956)

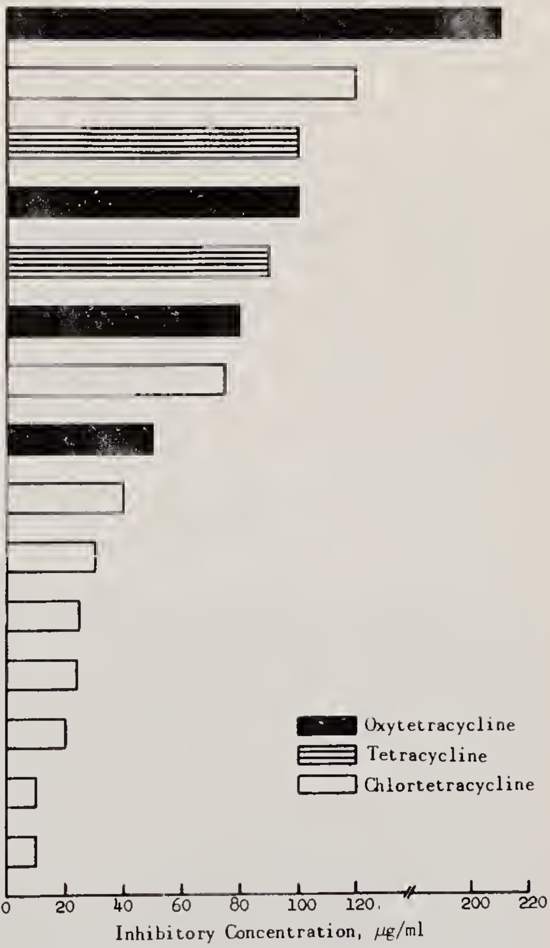

Fig. 7. Inhibition by tetracyclines (10-200 μg/ml).

From the present knowledge of intermediary metabolism it would appear that inhibition of reactions such as those of electron transport and D-glutamic acid utilization for wall synthesis are sufficiently critical and general among bacterial species that their inhibition would account for cessation of growth. However, the levels of antibiotics reported for significant effects in the *in vitro* system are about 50-100-fold the levels that inhibit growth of the organism;

hence, it may be necessary to invoke conditions *in vivo* that would allow for 100-fold concentration gradients of the antibiotic between the metabolic pools in equilibrium with these reactions and the extracellular medium. This condition would not appear to be too unlikely for serious consideration.

SUMMARY

1) Modes of action of the tetracyclines may be multiple, depending upon such factors as organism, medium, concentration, functional groups, and intracellular distribution of the antibiotic. The literature has been summarized covering activities of tetracycline antibiotics on chelation in relation to inhibition, enzymatic inhibition, and inhibition of oxidative and synthetic reactions of the cell. These data have been arranged and discussed not only with regard to level of organization of the systems involved, but summarized in terms of minimum reported active concentrations of antibiotic.

2) We have demonstrated an interference with the metabolism of endogenously synthesized D-glutamic acid. The mode of this interference is complex to analyze. Experiments reported here indicate, however, that accumulation of radioactive D-glutamic acid from C^{14}-acetate precursor takes place in broth of inhibited oxytetracycline-sensitive *E. coli* cells, but not in that of resistant cells. The quantity accumulating is a function of the concentration of the oxytetracycline and is linear within a small range of oxytetracycline concentrations (0-2.5 μg/ml). The C^{14}-glutamate accumulating was all of the D-configuration at an oxytetracycline level of 50 μg/ml.

3) Chlortetracycline and tetracycline have been found to produce broth accumulation of C^{14}-glutamic acid from 2-C^{14} acetic acid under similar conditions.

4) We have postulated a structural relationship between glutamic acid and the A ring of the tetracycline antibiotics which suggests consideration of the possibility of specific inhibition in the synthesis and metabolism of D-glutamic acid into cell wall materials. Such a relationship may also supply a *modus operandi* for interferences that have been demonstrated by others with glutamic acid oxidation.

5) The inhibition of at least two reactions studied (utilization of D-glutamic acid for cell wall synthesis, and electron transport) would appear to fulfill criteria for inhibition of essential processes in multiple biological systems. Levels of tetracyclines required for these inhibitions have not been titrated to their minima.

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ANTIBIOTICS AND ANIMAL GROWTH

James McGinnis

Washington State University

As you perhaps are aware, the earliest observations showing that addition of small quantitities of an antibiotic to chick feeds increased growth were made in 1946 at the University of Wisconsin. The observation was disregarded as being of practical value at the time because of the scarcity and high price of antibiotics. Other growth responses which might be considered to be of the same type as that obtained with antibiotics were made a year or two later when an arsenical was added to chick diets based largely on raw soybean oil meal. After the identification of vitamin B_{12} activity, it became obvious that growth responses were being given by substances which did not appear to be identical with any of the known nutrients. It was not until the early part of 1950 that it was established that a number of fermentation residues produced as byproducts of different antibiotic fermentations possessed similar activity in stimulating chick growth. Soon thereafter it was announced by Stokstad and Jukes that pharmaceutical Aureomycin gave a similar response. Since this observation in 1950, literally hundreds and hundreds of experiments on the growth-promoting effect of antibiotics for animals have been conducted and, it would almost seem, as many papers on the subject have been published. Unfortunately, when all of the work and all of the papers are considered, only a few basic and important facts seem to be firmly established. Number one is, of course, that antibiotics of various types such as penicillin, streptomycin, Aureomycin, Terramycin, bacitracin and many others appear to give a similar growth response when added at low levels to the diets of young animals. The second effect is that, even though less well established, the growth response observed in animals is mediated through an effect of antibiotics on microorganisms. When germ-free chicks were used, a growth response was not obtained to antibiotic supplements in two different laboratories. It is interesting that growth of the control germ-free animals was superior to that of comparable animals not germ-free and without antibiotic supplement. A third fairly well established observation is the variability that is obtained in responses to different antibiotics and even to the same antibiotic under different environmental or laboratory conditions.

Following the initial announcement that antibiotic supplements stimulated growth, many confirming reports were published. There were, however, some reports showing what appeared to be lack of confirmation. On further examination of these reports, the lack of agreement appeared to be due to conditions under which the experiments were conducted. In most instances, the laboratories from which these reports came, or in which the chicks were kept, were either new or had been cleaned and disinfected as thoroughly as was possible. It would therefore seem that, aside from the above main points, little has been established concerning the basic mechanism or mode of action through which antibiotics stimulate animal growth.

Perhaps it would be advisable to consider in more detail some of the proposals that have been advanced to explain the manner in which antibiotics stimulate growth. Among the first of these was that antibiotics inhibited microorganisms that produce toxic or harmful substances in the intestinal tract of the animal. Evidence in support of this was obtained in an early study with pigs which showed that antibiotic supplementation of the diet greatly reduced the number of *Clostridium perfringens* in the intestinal tract. This observation did not appear to be confirmed in studies that were conducted elsewhere with chicks and reported at a later date. However, since that time a report with germ-free chicks showed that inoculation with *Clostridium perfringens* depressed growth and that the depression was prevented by use of an antibiotic supplement. More will be said later concerning the role of this type of organism in response to antibiotics.

Another proposal was advanced to explain the increased growth as due to increased appetite, and as a consequence, greater feed consumption. In our laboratory we were able to demonstrate that an antibiotic supplement gave a growth response even when feed intake of experimental groups was equated. It should be pointed out, however, that the growth response was reduced when the feed intake of the antibiotic-supplemented group was restricted to that of the basal control. Another proposal that is based on stimulation of nutrient synthesis by intestinal microorganisms, in my opinion, has little support from a practical application standpoint. It has been amply demonstrated that diets deficient in many of the nutrients such as B vitamins are improved to a greater extent by antibiotic supplements than comparable diets containing adequate amounts of these nutrients. I do not believe we have yet been able to eliminate the need for an antibiotic supplement by nutrient fortification of the diet as such, unless it is assumed that a grass-juice supplement used in a number of experiments at Cornell University did not contain antibiotic substances. In this particular instance the grass-juice concentrate was as effective as Aureomycin in stimulating growth of turkeys, and a combination of the two was no more effective than either one alone.

Others have proposed that antibiotic additions to the diet increase growth by promoting more rapid and complete absorption of nutrients from the intestinal tract. This proposal does not seem to have much substantial support, since little or no difference has been obtained in the rate of absorption of specific nutrients such as phosphorus. There are many other proposals that have been offered to explain the effect of antibiotics, but so far the results in support of specific proposals do not appear to be overwhelming or even in agreement from laboratory to laboratory.

Perhaps it would be more prudent to assume that instead of a single explanation being sufficient to explain the growth-promoting effects of antibiotics, we will find many different modes of action in operation, varying with circumstances such as diet, environment, age of animal, type of animals, etc. There appears to be no reason to assume that a single mode of action will ultimately be found.

At this time I would like to return to a discussion of some of the observations that have been made in our laboratories during the past eight or ten years. Initially we observed that a number of antibiotics such as penicillin, Aureomycin, Terramycin, and streptomycin gave in many instances phenomenal increases in growth. This situation prevailed for a period of two to three years with perhaps some reduction in the magnitude of responses being evident toward the end of this period. At about the end of 1952, the response to antibiotics virtually disappeared in our experiments. The assumption was made, as has been suggested by others, that antibiotic use had "cleaned up" the environment, so to speak, and decreased the effectiveness of such supplements in stimulating growth. A closer examination after this situation prevailed for several years showed that maximum growth was not being obtained, with or without antibiotic supplements, when considered in terms of what was obtained in the early experiments. In our early work during 1949–1950 it was not uncommon to have turkeys average 650–700 g at four weeks of age. After the antibiotic responses had disappeared, we rarely saw groups of birds averaging over 500 g, and in most instances, they weighed less. In pondering the reasons for this observation we wondered whether the apparent loss in effectiveness of antibiotics might not be explained by development of resistance of the organisms against which antibiotics act to permit greater animal growth, or perhaps a change in the microbial population to a type that was growth-depressing yet insensitive to the antibiotics being used. At about this time we obtained some oleandomycin and erythromycin for use in our studies. These antibiotics had not been used previously in our laboratories. It might be well to point out at this time also that penicillin, Aureomycin, and Terramycin were used extensively in our studies, and in most studies penicillin was used routinely as a component of all diets. This practice is different from that in some laboratories where antibiotics are used only in experiments where they are being investigated. When the two new antibiotics were included as supplements, we were somewhat surprised at the magnitude of the growth response obtained. The weights of birds were more similar to those observed in our early studies than any which had been seen during the previous several years. Typical results are shown in Table 1.

Table 1. Weight Response to Antibiotics in Feed

Supplement	Level in mg/kg	Av. wt. (g) in 4 wks.
None	-	528
Procaine penicillin	4.4	535
Streptomycin sulfate	4.4	510
Terramycin · HCl	11.0	541
Oleandomycin phosphate	4.4	630*
Erythromycin thiocyanate	4.4	617*

We were curious to know what would happen if one of these antibiotics was used on a continuous basis in our laboratories over an extended period of time.

Erythromycin was selected for this study, and before the end of a year of usage, the response had virtually disappeared. The results of our experiments with erythromycin are shown in Fig. 1. It can be seen that by December, 1958 the

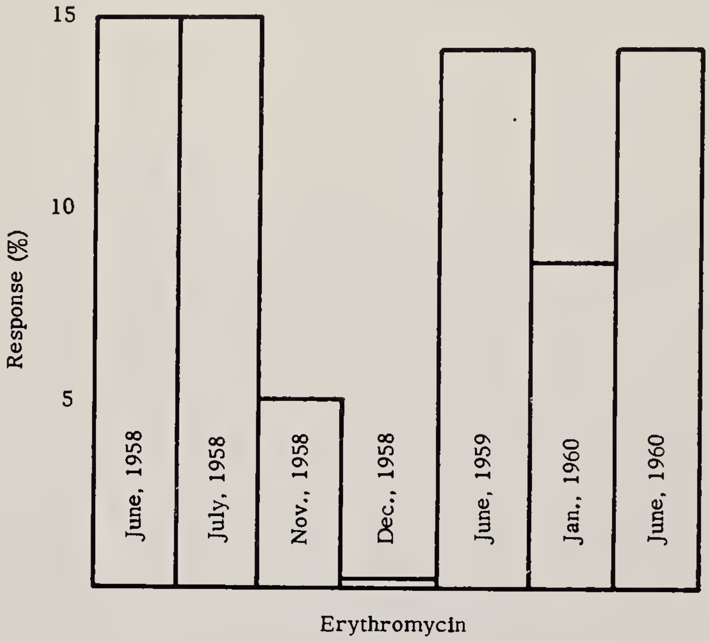

Fig. 1. Growth response to erythromycin antibiotic discontinued between December, 1958, and June, 1959.

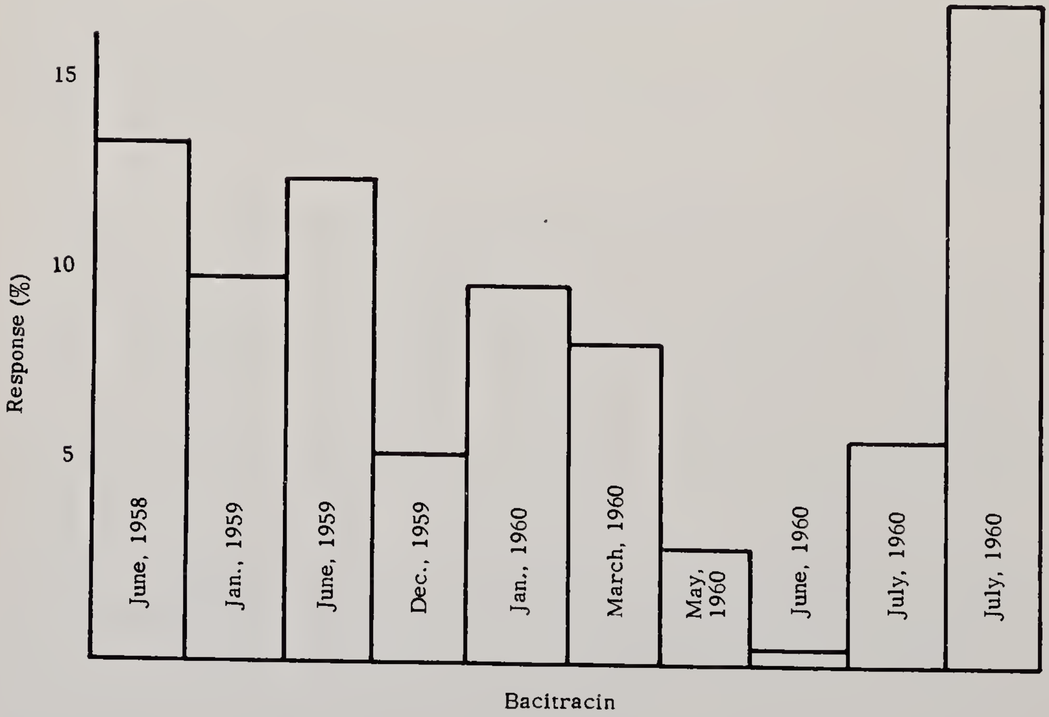

Fig. 2. Growth response to bacitracin.

response to erythromycin had disappeared. We then discontinued its use in our laboratories until June of 1959, at which time an excellent response was obtained.

At about the time the response to erythromycin was disappearing, we observed that bacitracin additions gave an excellent growth response. Some of the typical responses to this antibiotic are shown in Fig. 2.

It can be seen here that a fairly typical response was obtained over a period from the middle of 1958 into 1960. In one experiment we did observe a smaller than anticipated response. I would like to comment here concerning a change which we made in our routine operations in October of 1959. At this time, because of other considerations, we made the decision to include bacitracin in all experimental diets used in our laboratories in place of penicillin. There appeared to be a steady decline in the response to bacitracin during the following period of about ten months. I am sure you will be interested in the fact that while this was occurring, the response to penicillin increased. Likewise, you will perhaps recall from the data on erythromycin that during 1960 excellent responses to this antibiotic were also obtained. A comment on conditions that prevailed when the July experiments on bacitracin were conducted might be in order. At the time this experiment was run, no other animals were on experiment in our chick laboratories.

As a part of our department at Washington State University, we operate another laboratory located about 300 miles from Pullman, where a chick nutrition laboratory is also used. The practice in this laboratory is to conduct all experiments simultaneously and depopulate the premises when the experiments are terminated before starting new ones. The pattern which has been observed in response to antibiotics in our laboratory at Pullman has not been nearly as evident in the other laboratory.

I would like at this time to show you some of the results that have been obtained in our studies, using the same diets, chicks from the same sources, and keeping them in three different environments. In conjunction with these studies, we have made observations on growth, feed efficiency, microbial counts, and weights of intestinal tracts.

Table 2. Average Weight (g) in Four Weeks

Treatment	Location		
	Old	New	Floor
Control	283	306	257
Penicillin	287	305	270
Bacitracin	304	324**	279

You will be particularly interested in the marked influence that environment has on results obtained (Table 2). It has been our general observation that chicks raised on litter floored pens do not grow as well as those kept in battery brooders. The results which we obtained on *Clostridium* counts from chicks kept in the three different environments provide a basis for interesting speculation (Table 3). Us-

Table 3. *Clostridium welchii* Mean Plate Number ($\times 10^{-4}$)

Treatment	Location		
	Old	New	Floor
Control	4.8	152.7	885.2
Penicillin	14.4	61.7	126.7
Bacitracin	6.6	14.0	191.1
Control (wire)	-	-	4.6

ing these data, it would be possible to agree or disagree with most of the publications on this subject, depending on the environment used. You will note that antibiotics had virtually no effect on the *Clostridium* in chicks from our regular laboratory. In contrast, it would appear that *Clostridium* counts in the floor pen were reduced as the growth response to antibiotics increased. It would therefore appear that the conditions under which chicks are kept has a marked influence on the microbial population of the intestinal tract and the response of this population to antibiotics. The data on intestinal weight are also interesting. During a prolonged period of time it was routinely observed that the weight of the intestine expressed as a function of body weight was uniformly decreased whenever a positive response to antibiotics was obtained. This is evident from the data in Table 4. During the past four or five months some pronounced variability in this measurement has been encountered, and in some instances we have observed growth responses with no change in intestinal weight, and vice versa. It would therefore appear that decreased intestinal weight, as such, does not necessarily correlate with a positive growth response to antibiotics.

Table 4. Average Intestinal Weight (g)

Treatment	Location		
	Old	New	Floor
Control	43.3	37.5	51.9
Penicillin	45.5	39.6	57.4
Bacitracin	38.7 *	33.8 *	51.9
Control (wire)	-	-	57.2

In conjunction with the experiments with chicks and the observations on intestinal weight, we have conducted a considerable amount of work from the histological standpoint to determine whether an effect of antibiotics on the intestinal mucosa could be detected. So far, these efforts have been largely unproductive from the standpoint of finding an effect of antibiotics that is consistently correlated with growth responses. Nevertheless, the observations made have been quite interesting. At one time it was thought that a greater number of mitotic figures or dividing cells was evident in the intestinal mucosa of birds fed antibiotics than in the unsupplemented controls. More extensive observations and counting have not yet shown a statistically significant effect of antibiotics on mitotic figures. We have also tried to correlate positive growth responses with numbers of goblet cells in the intestinal mucosa. The observations in this instance are not very consistent either.

We are continuing our work on the histology of the intestinal tract in hopes of finding some type of change that is correlated with response to antibiotics which will be of help in explaining how these materials increase growth of animals. We are also continuing our studies on the use of different antibiotics for varying periods of time and in various combinations, in an effort to obtain more information on factors causing the type of variability in response to antibiotics that is commonly observed.

PLANT DISEASE ANTIBIOTICS*

Thomas G. Pridham

Northern Regional Research Laboratory,* Peoria, Illinois

A considerable time has elapsed since concerted efforts were begun in the United States to discover and develop antibiotics for the control of plant diseases. Despite the wide experimental interest in such development, the numbers of commercial products available and their sale have not yet expanded as anticipated. Most materials discovered apparently failed in field testing or analysis beyond those stages relegated to initial laboratory screening. Many microorganisms, and for that matter extracts of higher plants and inorganic and organic chemicals, can be readily obtained that show antagonistic effects toward laboratory cultures of phytopathogens. Unfortunately, many factors control their ultimate practical use. Phytotoxicity, toxicity to animals, failure in greenhouse and field trials, incompatability with other fungicides or insecticides and herbicides, difficulty of isolation and purification, instability, cost, and possibly general resistance on the part of prospective users toward new chemotherapeutants—all probably have contributed to the slow growth of this part of the antibiotic industry.

In our own experience, we have obtained many cultures that, in the laboratory, antagonize a variety of phytopathogens. Of some 400 filtrates obtained with these cultures which were submitted for greenhouse tests to the Growth Regulator and Antibiotic Laboratory, Crops Research Division, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland, about 20% have shown evidence of disease control. Of these, about 20 show enough promise to warrant further study. Some of these undoubtedly are producing previously described antibiotics such as streptomycin, endomycins, nigericin, streptothricins, and pleocidins.

An evaluation of the literature leaves one with the impression that there are many synthetic inorganic and organic chemicals which will do the job of disease control as well as, or better than, antibiotics. One soon becomes aware that many of the synthetic chemicals are not suitable. Continuing reports, from year to year, of comparative trials with agricultural chemicals clearly stand as testimony to their inadequacies insofar as plant disease control is concerned. Hence, research to find new antibiotics, or to uncover new uses for previously described materials, is fully justified. This statement is particularly true because many antibiotics have been found to enter plants and move about in an apparently unaltered state. This finding continues to excite interest both from a fundamental and a practical standpoint in view of the fact that some of the antibiotics translocate downward. While antibiotics may never be able to compete to any great extent with the protectant-type chemotherapeutant, their systemic potentialities offer considerable promise for fulfilling certain needs in plant disease control.

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*This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

GREENHOUSE AND FIELD TESTS OF ANTIBIOTICS, 1959-1960

The literature detailing results of past research with antibiotics for plant disease control has been adequately reviewed elsewhere. The most recent summaries are by Dimond (1959), Pramer (1959), and Goodman (1959). Consequently, the material covered here concerns only information reported during the past year and a half. It is becoming more and more difficult to assess results reported in the literature on a comparative basis. The great variety of experimental conditions employed, the dependence of experimental results on the whims of weather and naturally occuring pathogens, and various other factors often serve to bewilder the reviewer. Evaluation of antibiotics as single entities becomes more difficult because these materials are being used in combination with other antibiotics, pesticides, stickers, spreaders, growth regulators, and wetting agents.

One point that continues to be particularly disconcerting has been the apparent disregard for precise terminology in referring to materials under test. In some instances synthetic organic compounds, having no apparent relationships with antibiotics in any sense of the term, have been blithely referred to as "antibiotics." In addition, trade names have been used rather indiscriminately to refer to the generic component of various formulations containing, in addition to the antibiotically active component, a variety of other materials. This has been particularly true of the antibiotics cycloheximide, oxytetracycline, and streptomycin, and of the various formulations containing these materials. More precise terminology of test materials and better definition of concentration levels in terms of the antibiotic component, is highly desirable. Thorough identification would seem to be of particular importance in studies concerned with the absorption, translocation, and systemic activity of these chemicals.

In the period from January 1959 to August 1960 there were few reports on new antibiotics tested in greenhouse and field experiments. About the only new materials noted are hortesin (Oliver *et al.*, 1959), nicetin (Lindner *et al.*, 1959), and sodium usnate (Ark *et al.*, 1960). Much work has been carried out with some of the more readily available antibiotics, as pure compounds and in various formulations. Formulations containing cycloheximide and its various analogues, oxytetracycline, phytoactin, and streptomycin have received considerable attention. The role of griseofulvin, on which much work has been done, has been reviewed by Brian (1960) and several independent reports on its activities were recently made (Table 1).

Russian researchers (*Mikrobiologiya* 4, 156–160, 1959) scheduled continued trials in 1959 with grisein (an antibiotic discovered in this country), phytobacteriomycin, arenarin, trichodermin, and a number of uncharacterized materials. The results of these studies are not known, but there have been no reports in this country on the effectiveness of grisein in plant disease control.

Table 1. Antibiotics, Used Alone or in Combination with Other Agricultural Chemicals, That Showed Some Degree of Activity in Controlling Plant Diseases, 1/1959-8/1960

Antibiotic	Disease Controlled	Reference
Antimycin A	Rice blast	Misato *et al.*, 1959
Blasticidin	Rice blast	Misato *et al.*, 1959
Blastmycin	Rice blast	Misato *et al.*, 1959
D-Chloramphenicol	Cucumber (tobacco mosaic virus)	Lindner *et al.*, 1959
DL-Chloramphenicol	Cucumber (tobacco mosaic virus)	Lindner *et al.*,1959
Chlortetracycline	Tobacco mosaic virus	Shimomura and Hirai, 1959
Cytovirin	Cucumber (tobacco mosaic virus)	Lindner *et al.*, 1959
Cytovirin	Pepper (potato virus Y)	Simons, 1960
Dextromycin	Tobacco mosaic virus	Shimomura and Hirai, 1959
Fermicidin	Tobacco mosaic virus	Shimomura and Hirai, 1959
Griseofulvin	Grain and grass smut and decay	Crosier, 1959
Griseofulvin	Spanish onion leaf blight and storage rot	Husain and Newhall, 1959
Hortensin	Tomato early blight	Oliver *et al.*, 1959
Kanamycin	Tobacco mosaic virus	Shimomura and Hirai, 1959
Mitomycin C	Tobacco mosaic virus	Shimomura and Hirai, 1959
Naramycin	Tobacco mosaic virus	Shimomura and Hirai, 1959
Nicetin	Cucumber (tobacco mosaic virus)	Lindner *et al.*, 1959
Oligomycin	Safflower rust	Thomas, 1959b
Oxytetracycline	Apricot crown gall	Ark and Thompson, 1960a
Oxytetracycline	Peach crown gall	Ark and Thompson, 1960a
Oxytetracycline	Olive knot	Ark and Thompson, 1960a
Oxytetracycline	Pecan gall	Cole, 1959
Phytostreptin	Cucumber (tobacco mosaic virus)	Lindner *et al.*, 1959

Antibiotic	Disease Controlled	Reference
Rimocidin®	Sesame damping-off and leaf spot	Thomas, 1959a
Trichodermin	Potato, tomato, and cabbage *Rhizoctonia solani* infections	Seiketov, 1959
Trichodermin	Wheat hard smut, fusariosis, and anthracnosis	Fedorinchik, 1959
Tetracycline	Cucumber (tobacco mosaic virus)	Lindner *et al.,* 1959
Tetracycline	Tobacco mosaic virus	Shimomura and Hirai, 1959
Virusin 1609	Rose and cucumber powdery mildew	Protsenko *et al.,* 1959
Sodium usnate	Cucumber, bean, and Lima bean downy mildew, bean rust, bean anthracnose, stone fruit brown rot	Ark *et al.,* 1960

Table 1 lists those antibiotics reported to show some degree of control of particular diseases under a variety of experimental conditions and in combination with one or more adjuvants. It must be emphasized that, generally, these antibiotics were tested alongside other pesticides and agricultural chemicals. In many cases the antibiotics were not the most satisfactory therapeutants and were not recommended for grower use. There were, however, recommendations for further study in certain instances.

The majority of the antibiotics listed in Table 1 were tested in only a few experiments. Those which were tested more extensively are discussed below.

Cycloheximide

Cycloheximide, generally used in commercial preparations, elicits some degree of control of shothole of chokecherry (Inman and Weihing, 1959); black spot of rose (Rosen, 1959); rust of safflower (Thomas, 1959b); leaf blight of hawthorn (Strong, 1960); tobacco mosaic virus infections of cucumber and tobacco (Lindner *et al.,* 1959; Shimomura and Hirai, 1959); smut of oats and stinking smut of wheat (Crosier, 1959); leaf spot, brown rot, and blossom blight of cherry (Ehlers and Moore, 1960; Klos, 1960; Lewis and Hickey, 1960; Szkolnik, 1960); blister blight of tea (Ram and Jayaraman, 1960); rust of Welsh onion (Hoshino, 1960); rust of cedar hawthorn (Himelick and Neely, 1960); ray blight of chrysanthemum (McFadden, 1960); dollar spot and copper spot of turf (Bachelder and Engel, 1960), *Helminthosporium* leafspot, fading-out, brown patch, rust, cottony blight, and dollar spot of turf (Kreitlow and Juska, 1960); blister rust of white pine (Anonymous, 1960); black knot on prunes (Lewis, 1959/60); and damping-off and sore-shin of cotton (Sinclair, 1960). Cycloheximide was the most active chemical of some 233 tested for inhibition of tobacco mosaic virus multiplication in cucumber. Only 7 μmoles (2 ppm) were required to give 50% inhibition (Lindner *et al.*, 1959). The antibiotic, in a solution containing 1000 ppm Tween 20, was brushed on the upper surfaces of 11-day cotyledons inoculated previously with a virus-carborundum suspension. Cotyledons were inoculated by rubbing their upper surfaces with a cheesecloth pad moistened with the virus-carborundum suspension.

Negative results, or undesirable effects that were of more relative importance than the degree of control attained, were obtained with cycloheximide in field and greenhouse tests against powdery mildew of cantaloupe (Marlatt *et al.*, 1959); brown rot and leaf spot of sweet cherry (Cation and Friday, 1959); *Mycosphaerella musicola* infections of banana (Anonymous, 1959); dormant bud death of sweet cherry (Cameron, 1960); head smut of field bromegrass (Crosier, 1959); *Botrytis* petal spot of chrysanthemum (McFadden, 1960); and large brown patch of turf (Bachelder and Engel, 1960).

Cycloheximide Analogues

Cycloheximide acetate was found effective in tests against oat smut and wheat stinking smut, but ineffective against head smut of field bromegrass by Crosier (1959). It also was reported to inhibit the multiplication of tobacco mosaic virus in cucumber (Lindner *et al.*, 1959).

Cycloheximide methyl hydrazone gave moderate inhibition of multiplication of tobacco mosaic virus in cucumber (Lindner *et al.*, 1959), but the parent compound was superior.

Cycloheximide oxime was reported effective against oat smut and stinking smut of wheat, but ineffective against head smut of field bromegrass (Crosier, 1959). The antibiotic analogue also controlled rust of cedar hawthorn, but was less effective than the parent compound (Himelick and Neely, 1960).

Cycloheximide semicarbazone was found effective in tests against brown rot and blossom blight of cherry (Ehlers and Moore, 1960); blister blight of tea (Ram and Jayaraman, 1960); blister rust of white pine (Anonymous, 1960); oat smut and wheat stinking smut (Crosier, 1960); rust of asparagus (Murakishi, 1959); and inhibited multiplication of tobacco mosaic virus in cucumber (Lindner *et al.*, 1959). It was ineffective in control of head smut of field bromegrass (Crosier, 1960); leaf spot of watermelon (Schenck and Crall, 1960); and scab and spot of avocado pears (Ruehle and Young, 1959).

Cycloheximide thiosemicarbazone was found to give marked inhibition of multiplication of tobacco mosaic virus in cucumber (Lindner *et al.*, 1959).

Phytoactin

Phytoactin has received considerable attention during the past several years. This antibiotic has been found effective against *Cercospora apii* in celery (Darby, 1959); tobacco mosaic virus in cucumber (Lindner *et al.*, 1959); scab of apple (Dobie, 1960; Groves, 1960; Klos and Tatter, 1960; Powell, 1960); powdery mildew of apple (Dickens and Henderson, 1959); rust, sooty blotch, and X-spot of apple (Groves, 1960); blister rust of white pine (Anonymous, 1960); and leaf spot and brown rot of cherry (Ehlers and Moore, 1960; Klos and Reban, 1960). Poor response was obtained in tests against bean root rot (Rackham and Vaughn, 1959).

Streptomycin

Used as the sulfate, the nitrate, or as a component of various formulations, streptomycin continues to attract the interest of plant pathologists. Most of the diseases against which the antibiotic has been used are bacterial blights, spots, and rots although the interesting effect of the antibiotic against the downy mildews has also received attention.

The antibiotic has provided some control of seed-borne smuts and seed decay of grains and grasses (Crosier, 1959); crown gall of apricot and of peach, and olive knot (Ark and Thompson, 1960a); wildfire of tobacco (Shaw *et al.*, 1959); downy mildew of broccoli (Natti, 1959, 1960); bacterial spot of tomatoes (Sowell, 1959; Stall, 1959); Lima bean downy mildew (Heuberger and Crossan, 1959); potato seed-piece decay (Crossan, 1960); walnut blight (Miller, 1959, 1960); crown gall of pecans (Cole, 1959); hop downy mildew (Maier, 1959, 1960); bacterial leaf spot of sesame (Thomas, 1959); canker of acid lime (Rangaswami *et al.*, 1959); pear blight (Ark and Thompson, 1960b); bacterial blight and leaf spot of chrysanthemums (Conover, 1959; McFadden, 1960); bacterial blight of celery (Thayer, 1960); blister blight of tea (Ram and Jayaraman, 1960); strawberry leaf scorch and postharvest rot (Williams *et al.*, 1960); bacterial spot and ripe rot of pepper (Chandler, 1960; Taylor, 1960); and early and late blights of potato (Gallegly, 1960).

On the other hand, lack of control, extreme phytotoxicity, or other undesirable effects were reported when streptomycin in various forms was used in studies to control hop downy mildew (Ogawa *et al.*, 1960); bacterial rhizome rot of bananas (Stober, 1959); potato seed-piece decay and black leg (Ruehle, 1959); *Alternaria* leaf spot of sesame (Thomas, 1959a); tobacco mosaic virus multiplication in cucumbers (Lindner *et al.*, 1959); celery early blight and bacterial blight (Thayer, 1960); dormant bud death of sweet cherry (Cameron, 1960); and *Cercospora* leaf spot and bacterial spot of pepper (Crossan, 1960; Taylor, 1960).

Other Antibiotics

Negative or inconclusive results were obtained with many antibiotics in tests against plant diseases: chlortetracycline, fungichromin, neomycin, oxytetracycline, tetracycline, penicillin G, and polymyxin were found ineffective in inhibiting the multiplication of tobacco mosaic virus in cucumber (Lindner *et al.*, 1959); 22 of 30 antibiotics were found ineffective in leaf culture tests against tobacco mosaic virus (Shimomura and Hirai, 1959); nystatin did not prevent preemergence damping-off in cucumber or peas (Tape, 1959); oligomycin and phytostreptin failed in tests against root rot of bean (Rackham and Vaughn, 1959; McAnelly, 1960); and Rimocidin failed to control fusarium stem rot of carnation cuttings or bacterial spot of sesame (Petersen and Baker, 1959; Thomas, 1959).

ABSORPTION AND TRANSLOCATION OF ANTIBIOTICS IN PLANTS

There now exists a number of excellent articles on absorption and translocation of antibiotics and other compounds that are to be recommended to researchers concerned with systemic antibiotics (Dimond, 1959; Pramer, 1959; Epstein, 1960; Crowdy, 1959; Mitchell, Schneider, and Gauch, 1960; Mitchell, Smale, and Metcalf, 1960). Methods useful in studying absorption and translocation of antibiotics are given by Gray (1958); Mehta *et al.* (1959), and Mitchell *et al.* (1958).

Despite the fact that the uses of antibiotics in plant disease control show most promise if these materials are used as treating agents for seeds or propagative elements of plants, or as foliar sprays and dusts, there is relatively little information available about absorption of antibiotics and their subsequent fate in the plant when administered via these routes. Most information concerning antibiotic uptake and translocation in plants has been obtained through root-uptake studies.

Seed Uptake

Absorption of antibiotics by seeds has been demonstrated many times. In most instances, the evidence is only of a presumptive nature based on inhibition of germination, phytotoxicity, or disease control effects. Because seeds treated with antibiotics might represent a practical device for control of some diseases, it seems that more definitive studies are in order. Kruger (1959) has reviewed research in this area and has studied the use of antibiotics as seed protectants to control seedling decay of groundnuts, cob-and-tassel smut of maize, and kernel smut of kaffircorn. Cycloheximide, filipin, Rimocidin, and Thiolutin showed considerable promise.

With the bioassay, paper-chromatographic, and chemical techniques now available, application of these methods to determine the fate of antibiotics in whole plants obtained from treated seed is indicated. Mehta *et al.* (1959) have carried out such studies with vancomycin. Definitive information of a similar nature for other antibiotics would be valuable.

Seed uptake of streptomycin has been demonstrated by Borders (1959) through phytotoxic effects noted with cabbage, and through disease protection in sesame by Thomas (1959a). Thomas also has presented evidence of antibiotic uptake and retention in sesame and safflower with Rimocidin, cycloheximide, oligomycin, and our crude F-17 antibiotic mixture (Thomas, 1959a, 1959b). Mehta *et al.* (1959) through bioassay and paper-chromatographic techniques have demonstrated uptake of vancomycin and its retention and subsequent presence in such plants as tomato, cucumber, watermelon, pea, Lima bean, and pepper.

Cutting Uptake

This means of application of chemotherapeutants, of course, is of importance in control of diseases of those plants that are propagated by cuttings, e.g., certain ornamental flowers. Pramer (1959) has summarized the literature in this area, and Kuchaeva (1959) has demonstrated the uptake and subsequent presence in leaves of chlortetracycline, penicillin, streptomycin, oxytetracycline, phytobacteriomycin, grisemin, erythromycin, tetracycline, viomycin, polymyxin, and bacitracin, as well as a number of unnamed preparations from streptomycetes in cuttings of oak and mountain ash.

Root Uptake

From a practical standpoint uptake of antibiotics via root systems is of less interest than uptake via seed treatment or foliar sprays, except possibly in the field of hydroponics. This viewpoint is especially true because of reports indicating the destruction and adsorption of antibiotics in soil. Also, incorporation of antagonistic organisms in soil as disease preventatives generally has not been successful. Nevertheless, the great majority of work done on absorption and translocation of antibiotics has been carried out with root systems of various plants. The early literature in this area has been reviewed by Pramer (1959), Dimond (1959), and Crowdy (1959). The information leaves no doubt that a number of antibiotics are taken up by the roots of plants and moved upward in an apparent unaltered state. Because of the relative ease of experimental methods, some definitive evidence has been obtained through bioassay and chemical techniques. Vancomycin apparently is taken up by Lima bean, tobacco, pepper, eggplant, pea, tomato, and cucumber (Mehta *et al.*, 1959). Pimaricin, streptomycin, griseofulvin, and components of the F-17 antibiotic mixture also move up in root systems (Dekker and Ark, 1959; Brian, 1960; Maier, 1960; Mitchell *et al.*, 1959).

The ease of experimental handling of root systems has suggested that they be used in preliminary screening studies directed toward discovery of antibiotics which can be taken up by plants. Probably everyone engaged in screening operations has considered this technique at one time or another. In an early phase of our program we made a preliminary study to determine whether root-uptake data might be suitable for selection of materials that could be screened with greater profit in the greenhouse. Admittedly, the experiment was crude in nature, conditions were far from ideal, and results somewhat inconclusive. Nevertheless, comparison of our results with those reported by Gray (1958) suggest that a more refined technique of root uptake might have promise.

In our study, 309 streptomycete culture filtrates were subjected to root-uptake studies. Young Pinto bean plants were carefully removed from potting soil, the root systems were washed with running tap water, and the roots were then submerged in small beakers containing culture filtrate. Absorption was allowed to proceed for 48 hr in the laboratory at room temperature. After that time, approximately 10-mm sections were cut at the first internode and placed on agar seeded with several test bacteria and molds as in the conventional paper-disc assay method. Of the 309 culture filtrates tested, 109 gave evidence of uptake and movement of antibiotic activity with zones of inhibition against at least one of the test organisms (*Agrobacterium tumefaciens* [E. F. Sm. *et* Town.] Conn; *Corynebacterium flaccumfaciens* [Hedges] Dows.; *Xanthomonas phaseoli* [E. F. Sm.] Dows.; *Xanthomonas stewartii* [E. F. Sm.] Dows.; *Alternaria solani* [Ell. *et* G. Martin] Sor.; *Ceratocystis ulmi* [Buis.] C. Moreau; *Fusarium oxysporum* Schlecht. f. *lycopersici* Sacc.; and *Mucor ramannianus* Moell.).

A number of cultures producing known antibiotics were used in our study. Known producers of raisnomycins, duramycin and F-17-C, streptothricins, chloramphenicol, candicidins, hydroxystreptomycin and rotaventin, moldin, actinorubin, oxytetracycline and Rimocidin, chlortetracycline and tetracycline, candidins, pleocidins, streptolins, actinomycetin, actinomycin, endomycins, streptomycin, eurocidin, fungicidin, levomycin, vulgarin, aureothricin, ehrlichin, oleandomycin, and chartreusin were all positive. Stem discs inhibited at least one of the test organisms. Since most streptomycete cultures produce more than one antibiotic factor, there can be only speculation as to which one or ones might have been absorbed and moved upward. It is of interest, however, that our results are in general agreement with those obtained by other workers using more precise techniques and purified antibiotics.

In a modification of our method, 14 of the filtrates were selected. At the end of the 48-hr absorption period, the whole plant above the roots and out of contact with the filtrate was mashed into a *brei.* Small bits of the *brei* were placed on agar seeded with the several test organisms. Of the 14, 9 gave evidence suggestive of uptake and an upward movement.

Of the 109 filtrates showing evidence of uptake, 58 were studied in the greenhouse by our cooperators, 34 of these showed some degree of control for at least one of the plant diseases under study. Of the 200 filtrates showing no evidence of root uptake, 52 were also tested, and 25 of these controlled to some degree at least one of the greenhouse test diseases. The results suggest that preliminary screening through root-uptake studies might not improve screening techniques. However, the relative crudeness of the laboratory experiment, along with the facts that some of the greenhouse diseases studied were incited by obligate parasites and that the greenhouse work is based on systemic activity of materials applied to above-ground parts of plants, suggest that more highly refined laboratory studies of this nature might be in order.

Stem Application

Antibiotics applied to the stems of plants also are absorbed and may be translocated. Mitchell *et al.*, (1959) demonstrated this effect in control of bean rust with our F-17 antibiotic mixture, and earlier with streptomycin in control of halo blight of beans (Mitchell *et al.*, 1952). This method probably has application in the treatment of large plants, e.g., trees. Ark and Thompson (1960a) demonstrated the practicability of stem application in their work with crown gall of apricot and of peach and knot of olive. Maier (1960) and Hidaka and Murano (1959a, 1959b) also used this technique with streptomycin. Cycloheximide also has been demonstrated to be absorbed and translocated upwards when applied to trunks of white pine (Anonymous, 1960).

Foliar Application

This method, by far, is one most generally used to apply chemotherapeutants to plants. It seems to be the most logical reference base on which to establish laboratory and greenhouse screening operations. Oddly enough, the fate within the plant, of antibiotics applied as foliar dusts or sprays, has been studied only to a limited extent. Recent excellent reviews concerned with fundamental studies on the fate of various chemicals, including antibiotics, that have been sprayed or dusted on plants have been presented by Mitchell, Schneider, and Gauch (1960), and Crowdy (1959). A number of antibiotics have been demonstrated to enter plants with concomitant translocation upwards, downwards, or laterally. These antibiotics include streptomycin, oxytetracycline, streptothricin, pleocidin, vancomycin, and griseofulvin. It has been discovered that some of these materials translocate downwards (Gray, 1958; Mehta *et al.*, 1959). Again, it would seem that with the techniques now well-known to antibiotic researchers, e.g., bioassay and paper-chromatography, this movement would be a most promising research area. Recent reports demonstrating the absorption of antibiotics applied to above-ground parts of plants are those of Mehta *et al.* (1959), Ark and Thompson (1960b), Rangaswami *et al.* (1959), Goodman (1959), Mitchell *et al.* (1959), Ogawa *et al.* (1960) and Maier (1960).

Sooner or later, anyone engaged in screening for antibiotics effective in plant disease control realizes the importance of methods that duplicate conditions actually experienced in practical use of chemotherapeutants, i.e., seed treatment, cutting treatment, and foliar sprays or dusts. Beyond that, it seems that methods are needed that simulate greenhouse or field conditions in the laboratory without requiring living, healthy, or infected plants. The development of laboratory "plantless" methods that would allow accurate and rapid anticipation of results obtained in field trials is highly desirable. Perhaps thorough knowledge of the physical and chemical characteristics of the above-ground parts of plants might offer clues. Or perhaps methods such as that described by Klotz and DeWolfe (1959), in which tests are run in fleshy fruits or tubers, would allow more rapid and accurate selection of promising materials in the laboratory.

Leaf Immersion

One device that researchers have used for relatively rapid screening is that of leaf immersion. Again, this method requires living plants. Gray (1958) studied the uptake of a number of purified antibiotics in this way. He also pointed out certain objections to the method.

Along with our root-uptake studies we examined leaf immersion in our screening program, using 96-hr filtrates of 274 screening strains. One primary leaf of young Pinto bean plants was immersed in a small vial of culture filtrate and held for 48 hr in the laboratory at room temperature. At that time, small sections were cut at the first internode and placed on agar seeded with various phytopathogens. The results indicated that 32 of the filtrates contained antibiotically active factors that were taken up by the leaf and that moved downward. The group of 32 positive materials contained filtrates obtained with known producers of actinorubin, endomycins, hydroxystreptomycin and rotaventin, streptomycin and streptothricins. The results again are only suggestive that the particular antibiotics entered the leaf and moved into the stem, because paper-chromatographic or chemical studies were not made. However, streptothricin, previously demonstrated to move downwards by Gray (1958), gave positive results in our work, although the experiment was not carried out under ideal conditions. Considerably fewer materials were active in this test than in the root-uptake experiment. Subsequently, 17 of the 32 active filtrates were tested in the greenhouse by our cooperators. Eleven of these gave evidence of control to some degree for at least one of the plant diseases.

At present the most logical system for handling large numbers of culture filtrates in a screening program should incorporate a method whereby filtrates could be applied to healthy plants growing under ideal conditions. Subsequent assay of various parts of the plant could be made to select those materials that enter the plant and move downwards or laterally. Study of such materials in plants infected with pathogens against which activity had been demonstrated, would seem to offer considerable promise.

SUMMARY

New laboratory methods are needed for selecting antibiotics that are suitable to control plant disease. Sooner or later, one is confronted with the fact that the ideal screening method must revolve around living plants under conditions simulating those occurring in nature. Efforts should be made to develop methods that allow rapid screening of relatively large numbers of test materials with living plants or more preferably with appropriate simulants. More attention should be given to determine the fate of antibiotics absorbed by seeds, taken up by cuttings, or applied to plants as foliar sprays and dusts by bioanalytical, paper-chromatographic, or chemical techniques.

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NEW ASSAY METHODOLOGY IN THE FERMENTATION INDUSTRY

Samuel R. Green, Convenor

Panelists: Helen R. Skeggs
R. C. Kersey
R. R. Barton
Samuel R. Green

INTRODUCTION

Samuel R. Green

Wallerstein Company, Staten Island, New York

This symposium will cover current aspects of fermentation methodology. We are indeed privileged to hear from such qualified people in the field of fermentation assays as Drs. Skeggs, Kersey, Underkofler, etc. We are, of course, all aware of the fact that progress in science depends, to a large extent, on the availability of methods and procedures suitable to test the various theories and hypotheses of our scientific personnel. Similarly, one of the major criteria we employ to evaluate the conclusions of our laboratory work is observing the appropriateness of the methods involved in assaying the reactions evolved during the course of experimentation.

During the course of this symposium, we shall attempt to cover the procedures and methods used in assaying antibiotics, microbial enzymes, vitamins (as exemplified by Vitamin B_{12}), and techniques employed in evaluating the efficacy of industrial cultures. It is the consensus of this panel that, on the termination of this symposium, we will once again confirm the fact that it is not so much that scientific facts change, but that our enlarging knowledge and methodology experience grows beyond them.

CURRENT ASPECTS OF THE VITAMIN B_{12} ASSAY

Helen R. Skeggs

Merck, Sharp & Dohme Research Laboratories, West Point, Pennsylvania

The fermentation industry has been responsible for much of the impetus that has been given to the use of microbiological assays. The facts that by fermentation large quantities of vitamin B_{12} could be produced and that in the early days the microbiological assay was the only readily available analytical method for assessing the fermentation products, led to the widespread use of the various vitamin B_{12} assays when they were in a precarious stage of development, and consequently to a great deal of confusion. Gradually, a better understanding of the sensitivity of the assay systems has resulted in improved technical skill which, together with increased knowledge of the chemical complexities of the vitamin B_{12} molecule, has clarified many of the early problems. Experience has taught us, however, that variations occur in microbiological assays regardless of our utmost effort to maintain stable conditions and that successful application of microbiological assay techniques requires eternal vigilence.

Over the years, we have remained loyal to the *Lactobacillus leichmannii* assay (Skeggs *et al.*, 1950), in the face of the reported advantages of *Euglena* (Hutner *et al.*, 1949) and *Ochromonas* (Ford, 1953) with respect to sensitivity and specificity. In part this was possible because we never had occasion to work with material where the pseudovitamins B_{12} were a problem, and the *L. leichmannii* assay was sensitive enough to answer our purposes. Furthermore, we kept learning much from this artistic performer and enjoyed the challenge of maintaining a peaceful coexistence with a hard taskmaster.

It would be heresy to discuss vitamin B_{12} assays without reiterating the fundamental requirement for extra care in cleaning equipment and avoiding contamination in every step of the assay. This may sound picayune, but it is only common sense when your test system is being asked to distinguish between 1 and 2 $\mu\mu$g of a big molecule that is characterized by its ability to bind to a variety of chemical substances. The majority of the irregularities that occur in the performance of assays can be traced to contaminated equipment or faulty technique.

A recent review by Shaw and Bessell (1960) has covered admirably the various methods, both chemical and microbiological, available for the determination of vitamin B_{12}. In discussing microbiological assay methods, they state: "An understanding of the limitations of the various test organisms and the nature of the sample is essential to ensure that the methods of assay and the sample treatment selected are capable of giving a specific and quantitative result." This is an admirable thesis in that it compresses into one statement a variety of factors that may affect the outcome of an assay. From the practical standpoint, however, it is not always possible to know in advance the exact conditions necessary for the assay of a given material—a problem that may lead to considerable lack of rapport between the person awaiting results and the assayist. The limitations of the test organism depend on human ability to provide satisfactory assay conditions and properly to interpret assay results. Each microbiological assay is predicated on the ability of an organism to respond to a single essential nutrient in a quantitative manner under the conditions of the test.

However, the test organism is a complex metabolic system, reacting not alone with the single substance being assayed, but responsive to the conglomerate mass of factors both within and apart from the assay system to which it is subjected. Many of the factors that contribute to the success of the microbiological assay have been established, and the assays provide us with remarkably sensitive analytical methods, so precise under controlled conditions that we are lulled into a false sense of security. These are not static chemical systems but dynamic biochemical systems that must be constantly evaluated for possible evidence of evolutionary changes.

As long as an organism is carried in the artificial environment of the laboratory, it is subject to adaptation. If the medium in which it is maintained is suboptimal with respect to a required nutrient, it may acquire the ability to circumvent the need for a previously required nutrilite. *Lactobacillus fermenti,* for example, does not require thiamine unless it is maintained in a thiamine-rich medium (Sarrett and Cheldelin, 1944). In the same manner, the presence of a substance not essential for growth may spare an enzyme system to the extent that a nonessential substance or one that previously could be synthesized, becomes, perhaps not essential for growth, but necessary for optimal growth. In continued subculture in skim-milk medium, *L. leichmannii* became dependent on calcium for maximal growth, whereas the parent strain has no calcium requirement, and a latent responsiveness to ribose nucleotides developed into a requirement not only for the nucleotide, but for pyridoxal phosphate and a nucleoside. Thus, the vitamin B_{12} assay medium had to be supplemented to comply with the induced requirements of the organism.

Considering the impact of the medium environment on the metabolism of the organism, it is not surprising that many organisms give better assay performance when, instead of the inoculum being grown on a mixture of crude peptones as opposed to a test system that is highly synthetic, the organism is conditioned to the assay medium prior to its use in the assay. By subculture of *L. leichmannii* in assay medium supplemented with vitamin B_{12}, it has been possible, not only to improve the uniformity of response to vitamin B_{12}, but also to increase the sensitivity of the assay system to a range comparable with that of *Euglena*—1.0 to 10 $\mu\mu$g per ml.

In conditioning the organism to the assay medium, however, we overlooked the fact that it is necessary to bear in mind the type of sample being assayed. Adaptation to the assay medium resulted in a test system extremely sensitive and efficient for the analysis of cyanocobalamin in a synthetic mixture, but most of our work involves the assay of biological fluids, principally serum, in which vitamin B_{12} occurs complexed with various substances. In the assay of serum, because of poor recovery of added B_{12} in the recommended extraction procedure of Rosenthal and Sarrett (1952), we had adopted the procedure of Boger *et al.* (1955), employing straight dilution and assay in the presence of serum proteins.

Implied in this technique was release of bound B_{12} during sterilization of the assay, which resulted in precipitation of the serum proteins. With this procedure normal sera assay in the range of 300 to 700 $\mu\mu$g and results are compatible with those obtained with *Euglena.* Using a culture adapted to the assay medium gave the same results, initially, as a culture carried in skim milk, which we regard as the most natural medium for the maintenance of lactobacilli, with the advantage of increased sensitivity so that lower levels could be detected.

During the past winter, our normal control serum range suddenly dropped below 200 $\mu\mu$g and it appeared that most of the patients under study were in a state of subclinical vitamin B_{12} deficiency. Table 1 shows that the deficiency state was improved when we assayed the sera with an organism transferred directly from skim-milk stock culture. This experience pointed out two fallacies in our approach to the assay of serum. First, the direct dilution of serum, depending on release of free B_{12} in the assay tube was an erroneous assumption and second, the ability of *L. leichmannii* to utilize protein-bound vitamin B_{12} is not as straightforward as it appears, but depends on enzyme systems normally present in the native environment of the organism that become latent on continued subculture in a synthetic medium. The immediate solution to the problem lay in limiting the period of exposure of the organism to synthetic media to conform to the period during which it retained its ability to use bound vitamin B_{12}. The ultimate goal, however, is to find an extraction system capable of quantitative extraction and conversion of all of the serum-bound vitamin B_{12} to free cyanocobalamin, and at the same time simple enough for practical routine use. Using a pH 6.0 phosphate-buffer cyanide procedure, we have been able to extract all of the activity present on direct assay in a clear supernatant without any sign, to date, of interference in the assay. In some instances the activity, as shown

Table 1. Effect of Inoculum Maintenance on Vitamin B_{12} Assay of Normal Human Serum

Patient	B_{12} Micromicrograms per ml	
	Daily Transfer*	Stock Culture†
I-1	208	446
2	47	230
3	174	566
4	197	538
5	168	492
6	183	539
7	157	540

*Inoculum carried by daily transfer in B_{12}-supplemented assay medium for 8 weeks.

†Inoculum transferred 24 hr before use to B_{12}-supplemented assay medium out of skim-milk medium.

in Table 2, is greater in the buffer extract than in the direct assay. Recovery experiments have been satisfactory, but an insufficient number of samples have been run for us to be confident of reproducibility. The data in Table 2 introduce a phase of our research work that is of great current interest. The BO samples represent control bleedings on normal dogs. The B_{12} level is low, and in these sera, the extraction procedure yielded higher results when the serum protein was removed. The B5 samples represent bleeding taken five hours after the intramuscular administration of, in the first two dogs, 500 μg cyanocobalamin, and in the last pair of dogs 500 μg hydroxocobalamin. Table 3 shows the average

Table 2. Effect of Cyanide Extraction on Vitamin B_{12} Determination in Dog Serum

Dog No.	Sample	Micromicrograms per ml		
		NO* Extraction	CN† Extraction	Ratio CN/O
1	BO	188	322	1.72
2	BO	528	600	1.13
3	BO	274	421	1.53
4	BO	167	230	1.37
1	B5	5,300	5,550	1.05
2	B5	4,280	3,820	0.89
3	B5	70,800	77,200	1.09
4	B5	56,900	70,900	1.24

*Samples diluted with water for direct assay.

†1 ml sample in 5 ml 0.1 M pH 6.0 phosphate buffer containing 0.1 mg/ml NaCN autoclaved 15 min at 120C; cooled and diluted to 50 ml. Supernatant assayed directly or diluted with water to assay range.

Table 3. Comparison of Serum Vitamin B_{12} Levels Following Intramuscular Administration of 500 μg Cobalamin Analogs in the Dog*

Hours after Administration	$\mu\mu$g Vitamin B_{12} Activity per ml		
	Cyanocobalamin	Hydroxocobalamin	P Value
0	360	280	
5	5,300	25,100	0.05
24	1,480	4,980	.001
48	1,050	2,570	.001
96	790	1,270	.025
168	480	780	NS

*Fifteen dogs were injected with each cobalamin in a series of 7 individual experiments. Results are expressed as geometric mean of individual vitamin B_{12} determinations on serum obtained at the indicated interval after injection.

results of repeated experiments comparing the blood levels of dogs following the administration of the two cobalamins (Skeggs *et al.*) to a total of 30 dogs. Consistently, the administration of hydroxocobalamin results in prolonged elevation of serum vitamin B_{12} levels as compared with cyanocobalamin, suggesting that hydroxocobalamin is more readily taken up by body receptor sites. Using the technique of Miller (1957), it was possible to demonstrate that in dogs administered hydroxocobalamin, more cobalamin activity was bound by serum proteins than following the administration of cyanocobalamin. Figure 1 shows that *in vitro,* there is a clear difference in the capacity of serum to bind the two cobalamins. Hydroxocobalamin is bound in proportion to the concentration present within the range tested, whereas cyanocobalamin is proportionately less bound as the concentration is increased. Thus, one explanation for the prolonged elevation of serum vitamin B_{12} levels following administration of hydroxocobalamin appears to lie in its preferential serum binding as compared with cyanocobalamin. The metabolic and therapeutic implications of these findings in dogs have yet to be evaluated. At this time we know only that a similar pattern

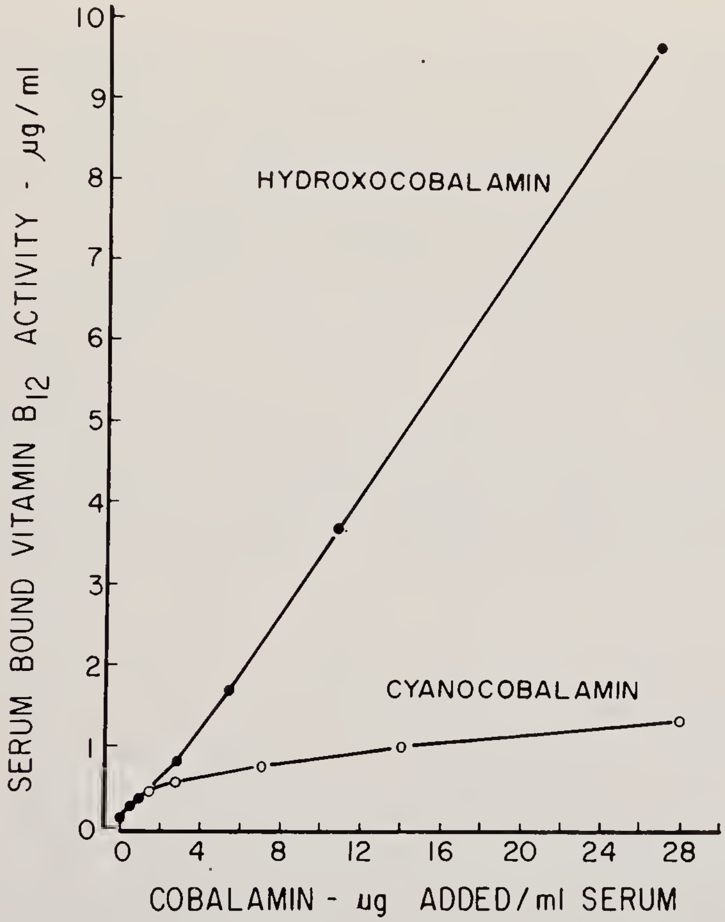

Fig. 1. Binding of cobalamins by normal dog serum. One ml of serum is incubated 30 min at 37C with 1 ml cobalamin solution of various concentrations. "Free" vitamin B_{12} is removed with 75 mg Darco G-60 and the supernatant diluted and assayed for vitamin B_{12} to determine the serum bound concentration.

of physiological retention of hydroxocobalamin also is observed in man (Boger, 1960; Glass *et al.*, 1960), and that tracer studies have confirmed our observations.

Thus, while we must admit to still incomplete knowledge concerning the factors that may influence them, the microbiological assay continues to contribute to our basic knowledge. We have come both to have great respect for the ability of these systems to follow precise biochemical pathways and to realize that deviation in their performance is usually due to the human element involved in their use.

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INTERPRETATION OF MICROBIOLOGICAL ASSAY DATA IN ANTIBIOTIC FERMENTATION

R. C. Kersey

Chas. Pfizer & Co., Inc., Brooklyn, New York

The new developments in antibiotic assays as they relate to fermentation have not been in the area of methodology. We still have the same old turbidimetric and plate technics. While it is true that refinements have been made in these procedures, they are of a general nature and their application is not limited to fermentation samples. What then is new? Have we made any significant advances since the early days of penicillin? The answer is certainly yes. There is a more sophisticated approach to analytical problems and there is a more enlightened interpretation of assay data.

The analytical problems relating to fermentation are or should be the mutual concern of the analyst and the fermentation chemist. I would like to stress the words "mutual concern." The research man usually possesses information which is vital to the analyst if he is to produce meaningful data.

Let us examine the researcher's problem of how to insure reliable data from the assay laboratory. First, and this is very important, the assay laboratory is not a dumping ground for the thoughtless disposal of large numbers of samples. If we are to realize the maximum information to be gained from an experiment, we must spend as much time planning the analysis as we did in devising the study. We must ask ourselves the following questions: What are we looking for? Should we sample at this stage or that stage? Will the sample tell us what we want to know? How closely do we need to pin down a value? Should it be a very precise determination or do we need only an approximation? What must the analyst know about this sample? How can we help him?

Microbiological assay methods are rarely, if ever, specific. The microbiological assay indicates the total biological activity of a sample of unknown composition. This is generally in terms of a concentration of a known standard.

When dealing with biological assays, remember this, nothing can be learned from a single one-point assay about interfering substances no matter how many times the assay is repeated. Unfortunately, there is no certain way of insuring assay validity. Assay invalidity, however, is clearly demonstrated by nonlinearity of response, departure from parallelism, and nonconsistency of results.

Let us consider for a moment chemical assays and see if they will offer us any help. We can now use a completely different approach by measuring certain chemical groups within the antibiotic molecule rather than measuring a biological activity. Obviously if we assay highly purified antibiotic preparations we find that chemical and biological methods are in very good agreement. This is just not so nor should we expect it with crude preparations such as fermentation broths.

Before we throw our hands up in disgust at the lack of agreement between biological and chemical test data, we should study it closely. It is just possible that the variability we see is the key to the solution of our fermentation problem. This discrepancy in assay data may be an important lead to what is happening in a fermentation. In other words this discrepancy may be trying to tell us something.

We will look at the microbiological, maltol, and streptidine assays of a streptomycin fermentation broth (Table 1). Our analysis might look something like this: microbiological assay 1000, maltol 1375, streptidine 1300 γ/ml. The question is, which figure is the right one? All of the figures are probably correct if taken in the light of what each test measures. Now assuming they are right, what do they mean? It is obvious that the samples contain free or at least some nonstreptomycin maltol. It also appears that there is free streptidine present. Are we able with this data to calculate the amount of streptomycin, maltol, and streptidine present? There is some question on the microbiological picture. Remember that the bioassay is not specific. It cannot differentiate between streptomycin A and B. Streptomycin B is about $\frac{1}{5}$ as active as streptomycin A biologically. Chromatography will tell us streptomycin B is actually present. If we find it, we may then quantitate it with the anthrone test for mannose. The important point here is the wisdom of not placing reliance in investigative work upon just one method of assay if another one of a fundamentally different principle is available.

Table 1. Potency of a Streptomycin Broth by Three Analytical Methods

Method	Potency
Microbiological	1000 γ/ml
Maltol	1375 γ/ml
Streptidine	1300 γ/ml

Another example of why you should not put all of your eggs in one assay basket is the detection of 6-aminopenicillanic acid. This precursor for the synthetic penicillins of today can be detected because the chemical and biological assays do not agree when certain precursors are left out of the fermentation medium.

I am sure that at some point in your careers you have submitted the same sample for biological assay at various estimates. Many times you probably found that the assay came close to the estimate. If your estimate was high, so was the resultant potency. If your estimate was low, so too the result. I am sure that the laboratory received a phone call that went something like this. "Look, what happened to one of my fermentation samples. I estimated it at 50 γ/ml and you reported a result of 60 γ/ml. On the same day I submitted it with an estimate of 200 γ/ml and you gave me a result of 170 γ/ml. One result is almost three times as high as the other. How can I make decisions with data like this?"

There is a very good reason for results of this nature. The difference in results is caused by nonparallelism of response between standard and sample (Fig. 1).

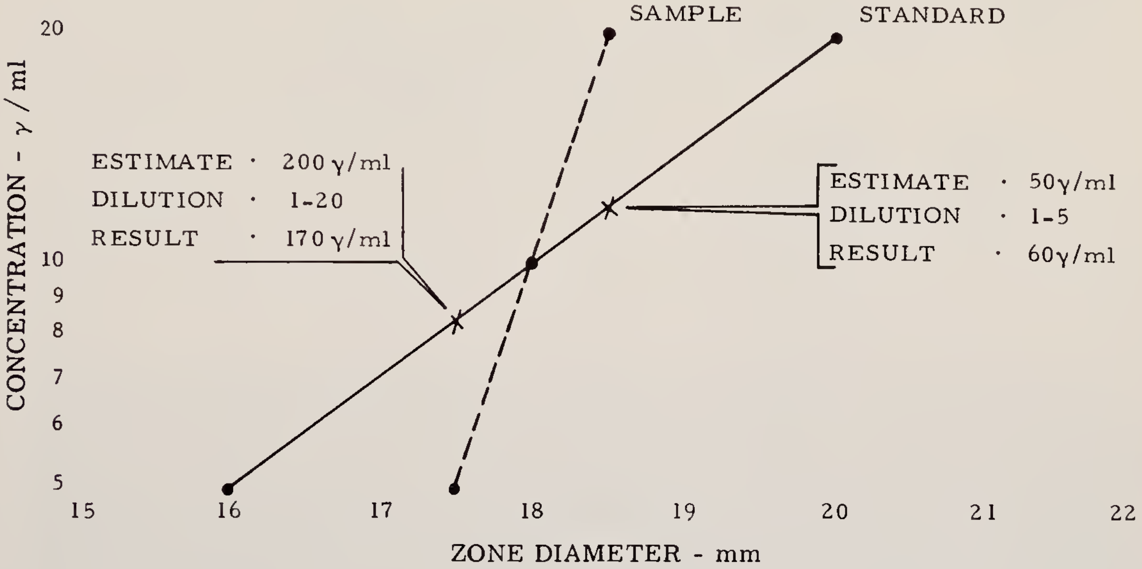

Fig. 1. Nonparallelism of response between standard and sample.

The solid line represents the response of the standard, while the broken line indicates the response of the sample. Assume that a fermentation sample has a potency of 100 γ/ml. If we estimate this sample at 100 γ/ml, the laboratory will dilute it 1 : 10 and we will obtain a response of 18 mm. At this point both curves will give the same result. But suppose we estimate the sample at 50 γ/ml. The laboratory will dilute it 1 : 5, the concentration of the assayed solution will be 20 γ/ml giving a response of 18.5 mm. On the standard curve, an 18.5 mm response is equivalent to 12 γ/ml solution. Since the dilution was 1-5 we obtain a result of 60 γ/ml on the original solution. Similarly if we estimate the sample at 200 γ/ml, the laboratory will dilute it 1 : 20. This solution contains 5 γ/ml and will give a response of 17.5 mm. On the standard curve a 17.5 mm response is equivalent to a concentration of 8.5 γ/ml. Since the dilution was 1 : 20 the laboratory will report a result of 170 γ/ml.

These results are not due to poor analytical techniques. Discrepancies of this nature point to nonparallelism of response between standard and sample.

So much for invalid assays. Now consider the variability that is inherent in the assay of any fermentation sample (Fig. 2). When we obtain results on duplicate fermentation flasks that differ by 30% or 40% we must remember that this is partly assay variability and partly fermentation variability. These two make up the total variability. Both assay and fermentation variability can be subdivided into their component parts.

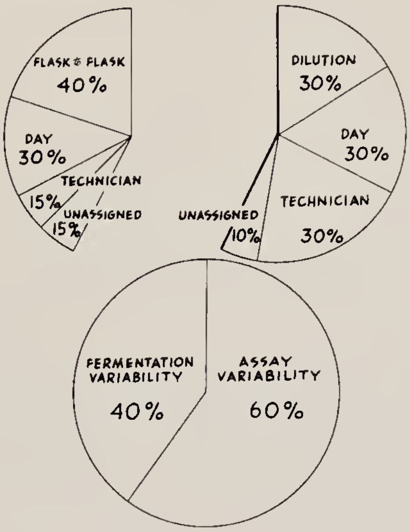

Fig. 2. Total variability is the sum of component parts.

Assay variability is produced by the technicians that handle the samples, by day-to-day changes in inoculum test curves and the like. Each of these factors can be studied to determine which one contributes most to the total assay variability.

The same is true of fermentation variability. It is the sum of its component parts. There are differences from flask to flask, from day to day, and from one technician to another. These parts can be studied to determine which one is responsible for the greater part of the variability.

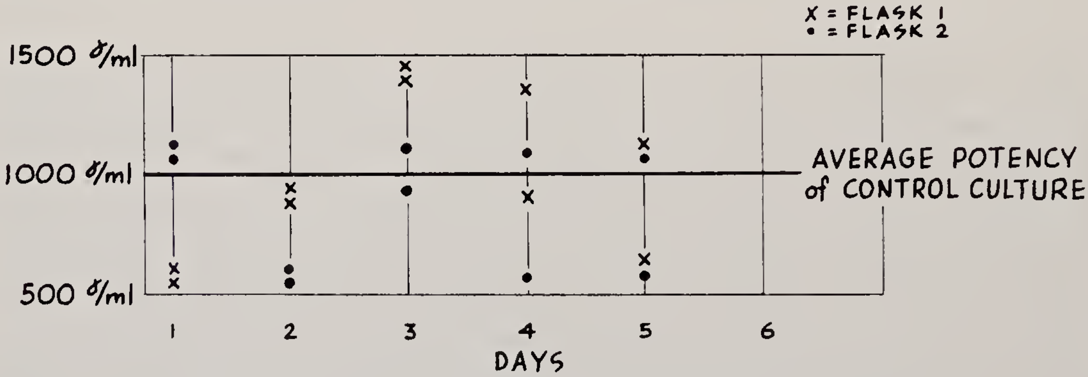

Fig. 3. Daily duplicate assays on two control fermentation flasks.

One simple way to investigate the two components of total variability is to submit duplicate flasks of a control culture on a daily basis and to request two assays on each flask (Fig. 3). Plotting the results each day around the average potency of the control culture will give you some indication of the source of variation.

On the first and second days it is fairly obvious that the assay is very reproducible. The flask difference is the greatest source of error. While on the fourth day, this is not the case. The difference between duplicate assays is just as great as the differences between duplicate flasks. On the fifth day the flask difference is negligible. Most of the error is in the assay. These figures can actually be used to quantitate the variability.

Once we know where most of the variability is we can concentrate on reducing it. One way to reduce variability is to increase the number of assays (Table 2). An average of 4 assays will certainly be more stable than a single value. By the same token an average of 10 assays will be more stable than an average of 4.

Table 2. Reduction of Assay Variability by Increasing the Number of Assays

No. of Assays	Variability
1	± 20%
2	± 14%
3	± 12%
4	± 10%
5	± 9%
10	± 6%
15	± 5%
20	± 4%

If we know the variability of a single assay is ± 20%, we can use this to estimate the variability of any number of assays. As a general rule the variability is reduced by the square root of the number of assays. The square root of 4 is 2. Therefore, if we run 4 assays the variability will be reduced by $\frac{1}{2}$; from ± 20% to ± 10%. After a certain point it is necessary to use very large increases in sample size to produce very small reductions in variability. The change in reproducibility from ± 6% to ± 5% may not be economical in the light of the number of assays required; 10 for the ± 6% figure and 15 for the ± 5% figure.

Estimates of variability such as this also serve another useful purpose. Suppose you have narrowed the field to 2 media that produce fairly high potency material. You are not sure whether they are different; one appears to be slightly better than the other. If you are really interested in knowing whether one medium produces a yield that is 10% higher than the other, don't try to find out by running duplicate assays. An average of 2 assays is only reproducible to ± 14%, so how could you possibly detect a real difference of 10% between your 2 media using such an approach. If you were to run 15 or 20 assays you would be using a more realistic approach to the problem since averages of 15 to 20 are good to ± 4 and ± 5% and would enable you to pick up 10% difference.

Similarly, if you are only interested in differences of 50% or greater, don't waste time and money by running more than one assay. You don't need more than a single assay to find differences this large.

So far we have considered the meaning of observed differences between assays of a different type and within assays of the same type. Now, I would just like to touch on one other new development in the fields of both fermentation and of assays. This pertains to the design of experiments.

For years we have used the one-variable-at-a-time approach in experimentation. If we are to study two media components at two different concentrations, we make up flasks on each one, a flask containing component A at the first concentration and a flask containing component A at the second concentration. Similarly for component B. We look at the result of each flask separately and decide whether we should discard the idea or pursue it with further experimentation. If we want to learn something about a medium which contained both A and B at the first or second concentration, we would have to make up four more flasks. So we need eight flasks to study the effects of A and B alone and A and B combined.

Actually we could study both variables and their concentrations at the same time. With four flasks we could obtain the same information as we did with the previous eight flasks (Table 3).

We can see the effect of A alone by looking at the totals of the A_1 and A_2 columns. A_2 looks interesting, 340 γ/ml as opposed to 240 γ/ml for A. In the same way, we can look at the effect of B alone by looking at the totals for the rows B_1 and B_2. There doesn't appear to be much difference between the high and low concentration of the B component.

Table 3. Study Two Variables Simultaneously and Save Money

		MEDIA COMPONENT A AT TWO CONCENTRATIONS		
		A_1	A_2	TOTALS
MEDIA COMPONENT B AT TWO CONCENTRATIONS	B_1	100 γ/ml	200 γ/ml	300 γ/ml
	B_2	140 γ/ml	140 γ/ml	280 γ/ml
TOTALS		240 γ/ml	340 γ/ml	

We can also obtain information on the combinations A_1 with B_1, A_1 with B_2 and so forth, by looking at each individual cell. Since A_2 looked somewhat better than A_1, we would naturally expect a difference, if it exists, to be in the A_2B_1 or A_2B_2 combination. This is exactly what happens. It is the A_2B_1 combination that gives a potency of 200 γ/ml, which is 40% to 100% better than any other combination.

This kind of design and interpretation is particularly useful when five or six variables and their various combinations are being studied. Large savings can be realized by this "factorial" approach.

In closing I want to point out the need for closer contact between the analyst and the fermentation chemist.

The analyst must make known the limitations of his assay. How precise is a single value? Is the assay a one point design and unable to detect nonparallelism of response or is it a multipoint design? How many assays would be required to detect a specified difference between two samples?

The fermentation man has an obligation to select his samples carefully not to overburden the assay laboratory. He should make use of some of the recent developments in experimental design when setting up his experiment. He must have a very clear idea of the magnitude of the response that he would consider significant. Finally, he must make intelligent use of the various analytical methods that are available.

METHODS OF ASSAY FOR MICROBIAL ENZYMES

L. A. Underkofler, R. R. Barton, and F. L. Aldrich*

Enzymology Research Laboratory, Miles Chemical Company, Division of Miles Laboratories, Inc.
Clifton, New Jersey

The determination of enzymes presents special problems in analytical chemistry because of the characteristics of the enzymes. Enzymes are proteins, usually present in small amounts, of high specific activity. With rare exceptions they cannot be determined by direct analysis, but must be identified and measured by means of the actions which they produce upon their substrates. Furthermore, most enzymes are labile, and subject to inactivation by unfavorable conditions of temperature, pH, or presence of inhibiting substances.

Since the determination of enzymes is dependent upon changes which the enzymes bring about in substrates, careful consideration must be given to the choice of substrates for enzyme assays. In many cases the substrates are quite complex materials, various proteins for proteases, starch for amylases, cellulose or cellulose derivatives for cellulases, fats or oils for lipases. When complex natural substrates are employed, further difficulties are experienced due to one of the most important characteristics of enzymes, their specificity. An amylase acts specifically on 1,4-glucoside linkages, for example, and its action will differ upon starches from different sources depending upon the relative amounts of amylose and of branched amylopectin, and the degree of branching of the latter. Even more complicated are the proteases which have action only upon specific peptide linkages between specific amino acids. The amount and availability of the required specific peptide linkages will differ between different proteins, such as gelatin, casein, and hemoglobin, and even between different batches of a single protein, for example casein, prepared in slightly different manner.

The action of enzymes is also affected greatly by environmental conditions. The rate of enzyme action increases with increasing temperature, but so also does the rate of enzyme inactivation. A suitable temperature must therefore be chosen for an enzyme assay procedure which gives rapid action on the substrate without significant inactivation of the enzyme during the period of incubation, and the temperature must be carefully controlled during this incubation period. Likewise the pH of the reaction mixture must be carefully controlled and maintained since enzyme activity varies greatly with only slight changes of pH. Suitable buffers which do not affect the activity of the enzyme but do control the pH of the reaction mixture must be carefully prepared and used. Other empirical conditions of mixing, agitation, and time of incubation must also be considered and controlled.

*Present address: Central State College, Edmond, Oklahoma.

Since the determination of an enzyme is dependent upon the action of the enzyme upon its substrate, various methods are possible for actual measurement. The amount of substrate left unchanged may be determined after suitable reaction periods. An example is determination of residual hydrogen peroxide in catalase assays. More often the amount of reaction product produced after suitable reaction periods is measured. Examples are the determination of reducing sugars in amylase assays, the determination of fatty acids in lipase assays, and of gluconic acid in glucose oxidase assays. Another possibility is the measurement of physical or chemical changes produced in the substrate, such as reduction of viscosity of starch or gelatin in amylase or protease assays, the change in iodine coloration of starch and dextrins in amylase assays, the increase in soluble nitrogen or amino nitrogen in protease assays.

The results of enzyme assays are expressed in numerous ways. Frequently units are established which represent the amount of substrate changed in a definite time under the specific conditions (pH, temperature, buffer, concentration) of assay, or the amount of product produced under the definite conditions. Sometimes the rate of change produced by the enzyme is not constant over a considerable period of time, being influenced by the concentration of the substrate and/or by the concentration of products during the reaction period, and possibly by progressive inactivation of the enzyme. For such enzymes the results of enzyme assays may be advantageously expressed in terms of the initial rate constants for the enzyme action.

It seems worthwhile to illustrate the general principles discussed briefly above by reference to specific assay procedures which have been developed and are used for measuring the activity of certain well known enzymes.

Procedures for the assay of amylase are very numerous, and it would almost seem that each investigator studying amylases or their action on starch has developed his own modifications. These numerous methods, however, can all be categorized in three classes: (1) Liquefaction of starch pastes, as determined by viscosity reduction; (2) dextrinization of starch as measured by iodine color reaction; and (3) saccharification of starch as measured by reducing sugar production. The methods based on reduction of the viscosity of starch pastes, such as that of Jozsa and Johnston (1935), in which the results are expressed as Liquefons units, have the serious disadvantage of great difficulty in producing starch pastes which have reproducible viscosity characteristics. Extreme care and elaborate equipment are necessary to surmount these difficulties, and such procedures have therefore found favor only for quite restricted purposes. The methods based on dextrinization are relatively simple, and are essentially modifications of the original method of Wohlgemuth (1908). By these procedures, the color produced by addition of dilute iodine solution to starch pastes upon which the enzyme is acting is observed. The changes in color resulting as the starch is progressively degraded is a measure of the course of the enzyme reaction. Various investigators have used visual matching of the color produced at different degrees of conversion. To eliminate the variability in judging the color end point, permanent color standards (Redfern, 1947) have been widely adopted for visual comparison, and certain investigators have found it advantageous to determine the end point by a photoelectric colorimeter. The saccharification method is requisite for determination of β-amylase, and is also advantageous for α-amylase where the degree of sugar production, rather than dextrinization, is of paramount interest. Various modifications are employed, in which different empirical conditions for incubation of the enzymes with starch are employed, and the sugar produced determined by any suitable method for measuring reducing sugars, including the several available copper reduction methods, ferricyanide reduction methods, and iodometric methods.

Undoubtedly the most complicated systems which are involved in enzyme assays are with the proteases. This is due to the complexities of the natural protein substrates, and the specificities of the proteases for particular peptide linkages. The situation is further complicated for most proteolytic enzymes in that, with the exception of a few which have been highly purified and crystallized, most available proteases are mixtures of several proteolytic enzymes which differ in specificity.

A useful method for protease determinations is based on the reduction of viscosity of gelatin solutions (Nickerson, 1952). Viscosities are measured with suitable viscosimeters after incubation for a definite time, or preferably at several times during the digestion period. Another widely used method is the formol titration procedure of Sørensen (1907). Formaldehyde is added to the substrate-enzyme mixture after digestion, and an aliquot titrated with standard alkali either with a pH meter or phenolphthalein indicator. Formaldehyde is added to another portion of substrate prior to addition of the enzyme, and an aliquot titrated. The differences in the titrations are a measure of the increase in amino groups resulting from the proteolytic action of the enzyme.

Among the commonest methods for protease assay using natural substrates are the procedures based on digestion of casein or hemoglobin. Again, numerous modifications of these procedures are used. In principle, the enzyme is allowed to act upon the buffered protein substrate under appropriate conditions for a suitable time. The reaction is stopped and unchanged protein precipitated at low pH, usually by addition of trichloroacetic acid. The amount of unprecipitated hydrolytic products is a measure of protease activity. This soluble nitrogen may be determined in aliquots of the filtered digest by Kjeldahl analysis, colorimetric determination by the procedure of Anson (1938) with the phenol reagent of Folin and Ciocalteu (1927), or spectrophotometrically in the ultraviolet region as described by Kunitz (1947).

For highly purified proteases, such as crystalline trypsin and crystalline papain, simple synthetic substrates are finding favor for protease assays. It is necessary to employ a substrate possessing a peptide linkage for which the enzyme has a specific action. For crystalline trypsin and papain, benzoyl-L-argininamide has found wide use. However, such synthetic substrates are usually of no advantage or risky for use with commercial enzymes where several proteases of different specificity and type of action (endo- or exo-peptidase) are present.

For the assay of proteolytic enzymes for control or standardization of products, the most convenient procedures, gelatin viscosity reduction, formol titration, or casein or hemoglobin digestion, may be employed. Using any of these procedures, standardization of a commercial microbial protease is possible, which will then give good reproducible results in use. However, great caution must be used in comparing enzymes from different sources upon the basis of a single assay procedure. Table 1 shows assay results obtained on several different enzymes by the different procedures. Note that the relative potencies of the different enzymes vary with the procedures. Great caution must also be exercised in anticipating the effectiveness of a protease for any particular end use with a substrate differing from that used in the assay. An important example is the use of proteases in bread baking. Enzymes from *Aspergillus oryzae* are permitted by our federal bread standards, and a hemoglobin assay procedure has been adopted by the cereal industry for standardizing the fungal proteases employed. However, experience has shown that enzymes derived from different strains of this organism, or produced by different culturing procedures, may vary markedly when tested by bread-baking methods. The only true way to evaluate the enzyme for this use is by bread-baking tests. When an effective enzyme preparation is found by such practical evaluation, it is then possible to correlate its efficacy with the hemoglobin assay method, and employ the latter for standardizing the enzyme for actual use. However, the organism strain and conditions of manufacture must be rigidly controlled if the assays by the hemoglobin method are to represent actual performance in baking.

Table 1. Assay Results for Some Proteases by Four Different Procedures

Enzyme	Hemoglobin Digestion, HU/g	Casein Digestion, NU/g	Formol Titration (gelatin), GDU/g	Viscosity Reduction (gelatin), VU/g
Bacterial protease	4,990	950	6,346	2,448
Fungal protease	53,100	202	310	112
Bromelain	8,660	933	1,680	1,099
Papain	8,640	776	1,205	784

DEVELOPMENT OF ASSAY METHODS

For control purposes, enzyme assay procedures should be rapid, accurate, and reproducible. In industrial usage, the relationship between an assay result and the amount of enzyme to use for a specific purpose is usually quite empirical. Therefore, once an assay procedure has been established for the standardization of an enzyme preparation, modifications are not readily accepted. Any change in the method must not affect the type of enzyme action measured. The modification will also have to produce the same numerical value as did the procedure being supplanted. Otherwise it will be necessary to reestablish the relationship between enzyme potency and end usage. However, it is desirable to improve accuracy and reduce the time involved in assay procedures for control purposes, and examples of how such improvements have been made in two assay procedures used routinely in our laboratories will now be presented.

Northrop Casein Digestion Assay

One of the established assays used to standardize bacterial protease, pancreatin, papain, and other proteolytic enzymes is the Northrop casein digestion method. This procedure is tedious and is subject to considerable variation. Basically this assay is run by digesting casein at pH 8.0 and at 40 C. Samples are taken at 15 min intervals and undigested casein precipitated by lowering the pH to about 4.0 and then filtering. Kjeldahl nitrogen determinations are run on the clear filtrate to determine the extent of hydrolysis. The numerical assay value depends on the time required to digest 40% of the casein, the unit being defined as the amount of enzyme which produces 40% hydrolysis in 60 min. To obtain reasonably good duplication of results, enzyme sample sizes are selected that will give 40% hydrolysis in about 35 min. Relatively small differences in the time required to achieve this 40% hydrolysis result in considerable variation in assay values. Experienced analysts frequently get variations of 15% or more between replicate assays of the same sample. Because of the many steps involved, it is possible for one person to run only four assays per day with this procedure.

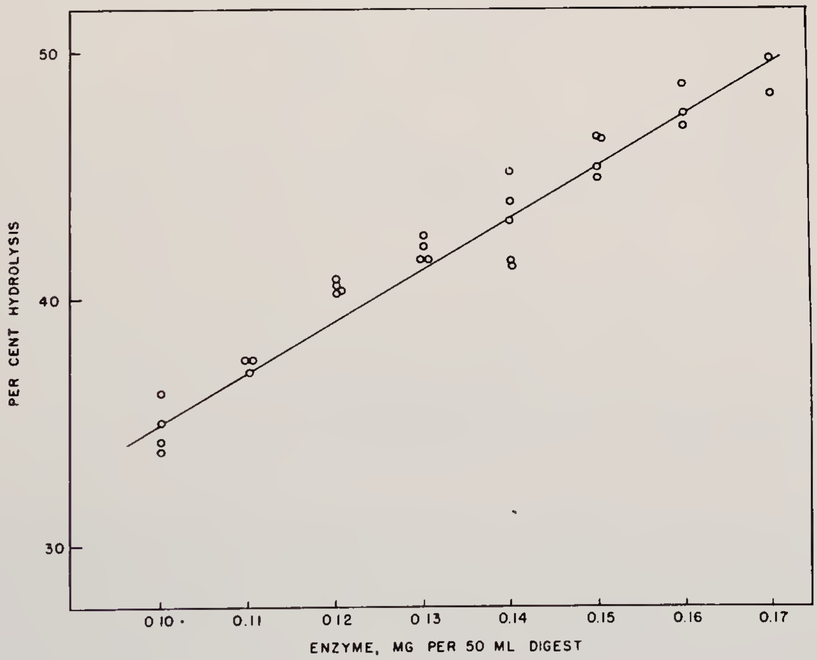

Fig. 1. Relation between enzyme concentration and per cent hydrolysis.

In developing an improved procedure, to avoid changing the type of proteolysis being measured, the same substrate and pH were used in the modifications made in the procedure. However, the first step in simplifying this assay was to reduce the amount of substrate for an assay from 1000 ml to 50 ml and to proportionally reduce the enzyme concentration. The next step was to use a fixed time of 35 min for the reaction. This time could be used, as it was demonstrated (see Fig. 1) that good linearity exists between extent of hydrolysis and concentration of enzyme. The individual points used in constructing this curve were the averages of four separate determinations.

To determine the amount of enzyme required to give exactly 40% hydrolysis in this time, two enzyme levels were employed for each sample, one level at about 20% higher concentration than the amount expected to give the desired degree of hydrolysis, and the other at about 20% lower concentration. The hydrolysis values obtained were then plotted against enzyme concentration and connected by a straight line. From this line the amount of enzyme to produce 40% hydrolysis could be determined.

The establishment of a fixed reaction time resulted in a significant improvement in accuracy, but did not result in an important saving in time. It was still necessary to measure hydrolysis by a time-consuming Kjeldahl determination, and to use at least two enzyme dilutions per assay.

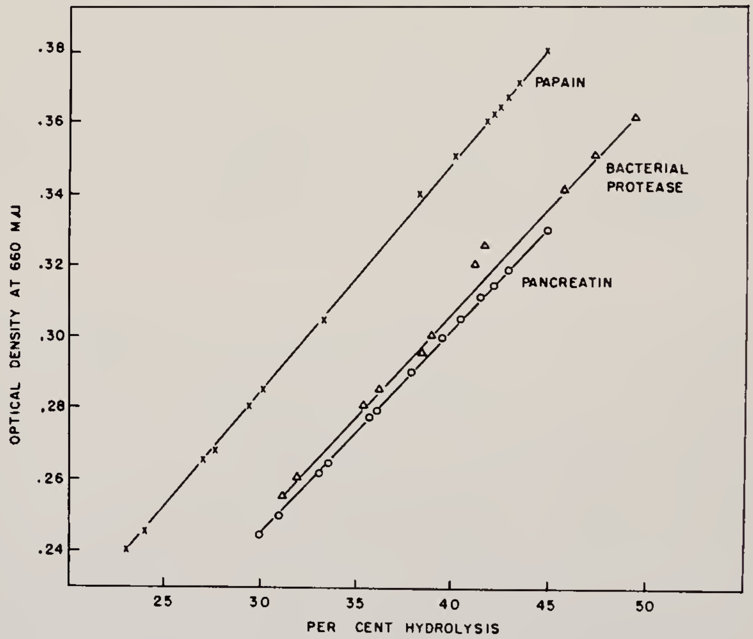

Fig. 2. Relation between optical density readings and per cent hydrolysis.

To reduce the time required it was necessary to find a shorter method for measuring the extent of hydrolysis. Measurement of the optical density of the filtrates at 275 mμ was found to be unsatisfactory as linearity between these measurements and enzyme concentration was not apparent. Use of the Folin-Ciocalteu (1927) phenol reagent, which reacts with materials containing tryptophane and tyrosine, was investigated. From Fig. 2 it can be seen that the relationship between per cent hydrolysis as measured by Kjeldahl nitrogen determinations and optical density readings at 660 mμ for digest filtrates treated with the phenol reagent was linear. However, there was a different relationship for each of the three enzymes used. This is due to the difference in specificities of these enzymes which resulted in the release of different residues from the casein substrate. Since per cent hydrolysis is proportional to enzyme activity, it is evident that the spectrophotometric measurements could be used to measure enzyme potency. However, to obtain assay values of the same order of magnitude as the old method, it was necessary to use factors in the calculations. The values of these factors vary with the type of enzyme being tested and the spectrophotometer employed. In order to standardize the spectrophotometric procedure, it is first necessary to run the fixed time assay to determine the exact potency of a typical sample and to correlate the optical density measurements by the type of calculations shown in Table 2. Once the factors have been established, the assay procedure is reduced to the following steps:

Table 2. Calculations for the Fixed Time and Spectrophotometric Northrop Procedure

Fixed Time Calculation

$$NU/g = \frac{6000}{35 \times E_t}$$

Calculation of Factor

$$F = \frac{NU/g \times E_s}{A_{660}}$$

Calculation from Spectrophotometric Assay

$$NU/g = \frac{A_{660} \times F}{E_s}$$

NU/g = Northrop units per gram.
E_t = Enzyme concentration (mg/ml) required to give 40% hydrolysis in 35 min.
E_s = Enzyme concentration (mg/ml) used to obtain A_{660}.
F = Factor.
A_{660} = Optical density reading at 660 mμ.

1. Prepare a 0.2% casein substrate buffered to pH 7.4. If a few drops of toluene are added this substrate may be kept for several days under refrigeration with no deleterious effects.
2. Pipette 50 ml aliquots of the casein substrate into 125-ml Erlenmeyer flasks and equilibrate to 40 C.
3. Once temperature equilibration is achieved, add 1.0 ml of an enzyme dilution that will result in an optical density reading within the range of 0.200 to 0.400 at 660 mμ.
4. After exactly 35-min reaction time, add 25 ml of acetate buffer, pH 4.0, to precipitate undigested casein, and filter.
5. Mix 2.0 ml of filtrate and 3.0 ml of 1.0 N sodium hydroxide in a test tube. Then add, by blowing from the pipette, 1.0 ml of dilute phenol reagent directly into the center of the alkaline solution, swirling gently.
6. Read the optical density at 660 mμ after 5 min but not more than 20 min after adding the phenol reagent.

A comparison of the assay results between the old method, the fixed time assay, and the spectrophotometric assay are shown in Table 3. It is evident that the results obtained with the fixed time and the spectrophotometric methods are in close agreement.

Table 3. Comparison of Northrop Assay Values Obtained by Three Procedures

Bacterial Protease Sample	Northrop Assay Values, NU/g		
	Old Assay	Fixed Time Assay	Spectrophotometric Assay
1	734	654	622
2	376	399	438
3	497	573	571
4	961	912	929
5	832	840	825
6	578	479	470
7	332	317	309
8	567	543	500
9	514	431	425
10	226	265	235
11	270	275	254
12	436	393	410
13	211	219	223
14	242	200	209
15	432	399	404
16	259	287	296
17	312	272	275
18	586	463	487

Hemoglobin Assay

Fungal protease used by the baking industry is usually standardized by means of a modification (Miller and Johnson, 1951) of the Ayre and Anderson (1939) procedure using hemoglobin as a substrate. This method is very long since it requires a 5-hr digestion period followed by a Kjeldahl determination of the trichloroacetic acid — soluble nitrogenous material. Due to the excessive amount of substrate used (2.5 g per 55 ml of buffer and enzyme solution) the concentration of enzyme is extremely critical. Slight excesses or insufficiencies of enzyme will result in too much or too little hydrolysis for accurate results. To make the extent of hydrolysis even more critical, Miller and Johnson (1951) found it necessary to raise the amount of solubilized nitrogen to the $^3/_2$ power to achieve linearity over even a small range of hydrolysis.

As with the Northrop assay, improvements in the procedure could not introduce changes which would change the type of enzyme activity being measured. For this reason the pH of the reaction mixture, pH 4.7, was rigidly maintained. It was found that changes of as little as 0.2 pH unit would cause differences of as much as 30% in the calculated assay results.

However, it was found that reducing the concentration of the substrate to 0.8 g per 55 ml resulted in increasing the reproducibility of results, as shown in Table 4. When the smaller amount of substrate was used, more uniform results were obtained over a rather wide range of enzyme concentrations while there was a spread of about 20% in the results obtained with the higher hemoglobin level. Indeed, the results are beyond the range of usable accuracy with these concentrations of enzyme.

Table 4. Comparison of Results Obtained with Different Levels of Hemoglobin

Enzyme Concentration, mg/55 ml	Calculated Hemoglobin Assay Values, HU/g	
	0.8 g Hb	2.5 g Hb
2.5	31,800	34,400
3.0	30,100	36,600
3.5	31,400	38,800
4.0	31,100	40,800

The next step in reducing the sources of error and the time required to run an assay was the use of soluble substrate. This was accomplished by adjusting the pH of a 3.2% suspension of Bacto hemoglobin with NaOH to pH 10.0. Mixing 25 ml of this solution with 25 ml of a 0.1 M acetate buffer, pH 4.5, resulted in a substrate of the correct pH and hemoglobin concentration.

Table 5. Proportionality of Enzyme Concentration and Reaction Time

Time, hr	Enzyme Concentration, mg/55 ml	Soluble Nitrogen, mg per 10 ml filtrate
0.25	8.00	1.36
0.50	4.00	1.37
1.00	2.00	1.31
2.00	1.00	1.32
3.00	0.67	1.32
4.00	0.50	1.34
5.00	0.40	1.31

The possibility of diminishing the digestion time by proportionally increasing enzyme concentration with decreasing digestion periods was next investigated. As shown in Table 5, it was found that the extent of hydrolysis, as measured by Kjeldahl nitrogen determinations, was quite uniform. The 30-min reaction time was adopted as convenient since the relationship between enzyme concentration and extent of hydrolysis is proportional over this time interval.

While the changes investigated thus far would shorten assay time and increase accuracy, Kjeldahl nitrogen determinations were still necessary. To avoid this time-consuming procedure the use of the Folin-Ciocalteu phenol reagent was investigated. As can be seen from Fig. 3 the relationship was linear over a wide enzyme range. For accuracy, enzyme concentration should be such as to give an optical density reading at 660 mμ within the range of about 0.200 to 0.500. The upper level of 0.500 was established in a comparison of the values obtained with several enzyme preparations where it was found that optical density measurements as high as 0.600 were sometimes unreliable.

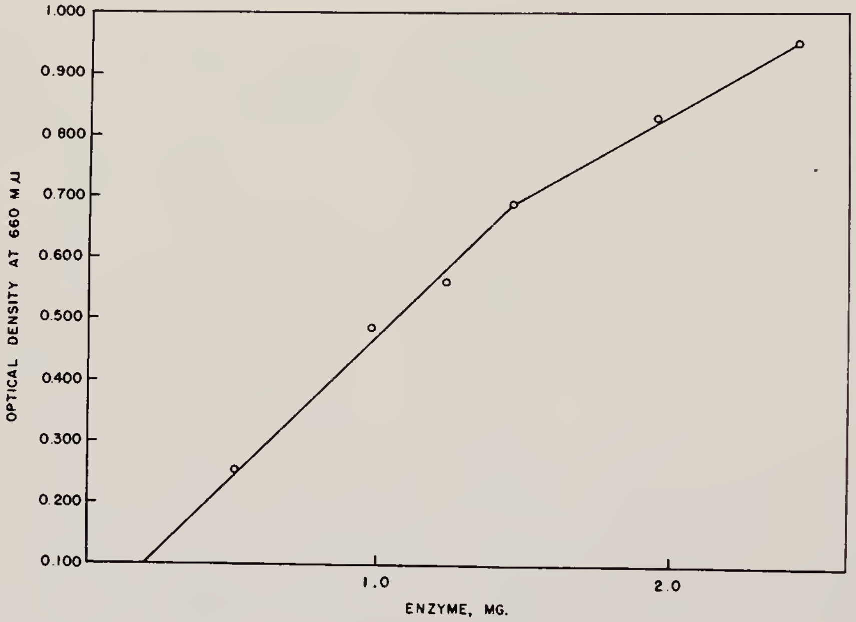

Fig. 3. Relation between optical density readings and enzyme level.

Thus with the adoption of a lower level and soluble substrate, the proper selection of enzyme concentration, and the use of the phenol reagent, a rapid and accurate assay could be achieved.

Table 6. Calculations Used for Determination of Hemoglobin Assay Values

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Standardization Assay

$$HU/g = \frac{\Delta N^{3/2} \times 6000}{E_t}$$

Calculation of Factor

$$F = \frac{HU/g \times E_s}{A_{660}}$$

Calculation from Spectrophotometric Assay

$$HU/g = \frac{A_{660} \times F}{E_s}$$

HU/g = Hemoglobin units per gram.
E_t = Milligrams enzyme used in standardization assay.
ΔN = Milligrams of soluble nitrogen; $\Delta N^{3/2}$ must be between 10.0 and 12.0.
F = Factor.
E_s = Milligrams of enzyme used to obtain A_{660}.
A_{660} = Optical density reading at 660 mμ.

However, the calculations made directly from the data would not give numerical assay values comparable to those of the original assay. To obtain such values it was necessary, as with the Northrop assay, to standardize the spectrophotometric method and obtain suitable factors. This was accomplished by running a 30-min assay with the Kjeldahl procedure to determine the increase in soluble nitrogen, then running the spectrophotometric method with the standardized sample. The calculations used in obtaining the factor necessary to obtain assay values of the desired magnitude are shown in Table 6. Once the factor has been established the assay procedure is reduced to the following steps:

1. Mix 25 ml of soluble hemoglobin and 25 ml of 0.1 M acetate buffer, pH 4.5, to give a substrate mixture of pH 4.7.
2. After equilibrating at 40 C add 5.0 ml of an appropriately diluted enzyme solution and incubate for 30 min.
3. After exactly 30-min reaction time, stop the reaction by adding 5 ml of 70% trichloroacetic acid solution.
4. After letting stand for 15 min filter off the undigested hemoglobin.
5. Run a spectrophotometric determination by mixing 1.0 ml of filtrate and 4.0 ml of 0.5 N NaOH, then adding 1.0 ml dilute phenol reagent by blowing directly into the center of this mixture and swirling gently.
6. Read the optical density at 660 mμ after 5 min but not more than 20 min after adding the phenol reagent.

Table 7. Comparison of Hemoglobin Values Obtained by Two Assay Procedures

Fungal Protease Sample	Hemoglobin Values, HU/g		
	Standard 5-hr Assay	Spectrophotometric Assay	Variance, %
A	55,600	58,400	5.0
B	30,400	33,300	9.5
C	24,300	26,700	9.8
D	109,800	105,100	−4.3
E	75,800	71,900	−5.1
F	57,200	59,400	3.8
G	65,400	68,300	4.4
H	33,300	34,600	3.9
I	78,000	76,000	−2.6
J	32,900	33,000	0.3
K	31,500	31,000	−1.6
L	32,300	32,400	0.3
M	84,000	80,500	−4.2
N	78,000	73,000	−6.4

A comparison of the results obtained by the tedious 5-hr assay and the short spectrophotometric assay are shown in Table 7. The maximum variation is within 10% with less than 5% for 72% of the samples.

SUMMARY

The special problems of enzyme assays due to the lability of enzymes, necessity of reliance on their action on substrates for determination, methods of measuring substrate change, and necessity of critical control of conditions were discussed briefly. Examples of different types of assay procedures for amylases and proteases were presented. Methods employed in modifying two assay procedures, based upon digestion of casein and hemoglobin, to improve their accuracy, reproducibility, and rapidity were described.

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THE EVALUATION OF STRAIN PURITY AND FERMENTATION VIGOR OF BREWERS YEAST

Samuel R. Green

Wallerstein Company, Staten Island, New York

Two of the major differences between beer and other fermentations are (a) the very high pitching rate (2 to 3 g of compressed yeast—equivalent to 15 billion yeast cells—per L of mash) and (b) the continuous re-use of the yeast crop derived from the beer fermentation (as many as 100 subsequent fermentations may be conducted without recourse to a fresh new "pure culture" of yeast).

In view of the high pitching rate and the continuous re-use of the yeast crop, it is evident that the fermentologist must have the tools necessary for the detection and enumeration of any small numbers of contaminating microorganisms in the yeast inoculum so that the brewer may be assured that the odor, taste, biological stability, etc. of the final product are not endangered by microbial contamination. In the same connection, any shift in the proportion of yeast strains in a known mixed culture of yeast must be known to maintain the fermentation and flocculation characteristics of the yeast during successive fermentations; thereby assuring, to a considerable extent, product uniformity. Similarly, the inadvertent introduction of new yeast strains or the occurrence of spontaneous mutations resulting in the replacement, either partially or completely, of the parent population must be recognized by the microbiologist. Finally, data concerning the fermentation vigor of the yeast must be available in order to evaluate the utility of the yeast after numerous fermentation generations.

While the use of hops, the low fermentation temperature employed, the acidity of the fermenting beer, the presence of alcohol, the anaerobic conditions prevailing during yeast fermentation, etc. tend to restrict the growth of contaminating microorganisms in beer fermentation, a limited extent of microbial contamination, nevertheless, does occur on occasion. The detection and enumeration of these bacterial and yeast contaminants in the presence of overwhelming numbers of culture yeast is rather difficult due to the fact that these contaminants *(Lactobacilli, Pediococci, Acetobacter, Flavobacteria,* and the fermentative, sporogenous foreign yeast) have growth temperatures, pH optima, and growth factor requirements similar to those of *Saccharomyces carlsbergenis* (lager yeast) and *Saccharomyces cerevisiae* (ale yeast). One would expect, therefore, that the use of the usual variations in culturing techniques, i.e., anaerobiosis, pH variations, deficient media, etc., which would tend to inhibit brewers yeast so as to allow the detection and enumeration of the contaminants, would also be likely to inhibit the growth of most, if not all, of the contaminating microorganisms mentioned above. Accordingly, the utility of materials extremely selective in their inhibition mechanism, the antibiotics, was investigated. The effectiveness of the antifungal antibiotic

Actidione (shown in Table 1) indicates that at a concentration of 2 ppm the growth of both *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* is inhibited (Green and Gray, 1950, 1951; Strandskov and Bockelmann, 1951, 1953). Of the hundreds of brewers culture yeast tested with Actidione not one has been found capable of growing in the presence of 4 ppm of Actidione, the concentration used in Wallerstein Laboratories Differential Medium. This concentration of Actidione is also capable of inhibiting the growth of culture yeast derived from compressed and dried bakers yeast, and from distillers yeast (Table 2). Studies with pure cultures of aerobic and anaerobic bacteria commonly found in brewers, bakers, and distillers yeast reveal that Actidione in concentrations up to 200 ppm fails to have any significant effect on the growth of these bacteria. Excellent recovery of lactic acid rods in the Actidione medium whether yeast is present or not has been noted (Table 3).

Table 1. Concentrations of Actidione Required to Suppress Growth of Various Ale and Lager Yeasts in Solid Synthetic Medium*

Brewery	Yeast Type	7 Days' Growth					
		Concentration: μg/ml of Medium					
		0	0.04	0.2	0.5	1.0	2.0
A_1	Ale	+	+	+	+	−	−
A_3	Ale	+	+	−	−	−	−
B	Ale	+	+	+	+	+	−
F	Lager	+	+	+	−	−	−
G_1	Lager	+	+	+	−	−	−
G_2	Lager	+	+	−	−	−	−

*Data from Green, S. R. and P. P. Gray, 1950. Wallerstein Laboratories Communications 13, No. 43:357.

Table 2. Concentrations of Actidione Required to Suppress Growth of Various Bakers and Distillers Yeasts in Solid Synthetic Medium*

Yeast	3 Days' Growth at 25 C				
	Concentration: μg/ml of Medium				
	0	0.5	1.0	2.0	4.0
Pressed Yeast A	+	+	−	−	−
Pressed Yeast B	+	+	+	−	−
Pressed Yeast C	+	+	−	−	−
Dried Yeast D	+	+	+	−	−
Distillers Yeast	+	+	−	−	−

*Data from Green, S. R. and P. P. Gray, 1951. Wallerstein Laboratories Communications 14, No. 47:289.

Table 3. Typical Plate Counts of Bacterial Cultures and Mixtures with Yeast*

Organism	Actidione Concentration	
	4 μg/ml	0
L. pastorianus	33,200,000	29,100,000
L. pastorianus and Brewers Yeast	28,900,000 (bacteria only)	40,800,000 (all yeast)

*Data from Green, S.R. and P.P. Gray, 1951. Wallerstein Laboratories Communications 13, No. 43:357.

Another antifungal antibiotic, Mycosubtilin, in concentrations of 10 ppm, proved to be useful also as a means of enumerating bacterial contaminants in brewing materials (Table 4).

Table 4. Concentrations of Mycosubtilin Required to Suppress Growth of Various Ale and Lager Yeasts in Solid Synthetic Medium*

Brewery	Yeast Type	7 Days' Growth						
		Concentration: μg/ml of Medium						
		0	1	5	10	20	25	50
C	Ale	+++	++	±	–	–	–	–
D	Lager	+++	++	±	–	–	–	–
G	Lager	+++	++	±	–	–	–	–

*Data from Green, S.R. and P.P. Gray, 1950. Wallerstein Laboratories Communications 13, No. 43:357.

Since brewers yeast may be contaminated with both Gram-positive and Gram-negative bacteria, it is of interest that phenylethyl alcohol in concentrations of 3000 ppm inhibited the growth of both Gram-negative rods and brewers yeast (Table 5), thus enabling the estimation of the Gram-positive bacteria contained therein (Strandskov *et al.*,1953). Additional studies have suggested that such antibiotics as penicillin, tyrothricin, erythromycin, etc. could be used to limit the growth of Gram-positive bacteria so as to permit a count of the Gram-negative bacteria in brewing materials (Bockelmann, 1954).

While the detection of minute numbers of bacteria associated with brewers yeast can be quantitatively estimated, the detection and estimation of undesirable foreign yeast genera and species, however, still presented a formidable problem. It is evident, of course, that the identification of certain yeast types is fairly simple. Thus, for example, the presence of aerobic, nonfermentative, foreign yeasts such as *Candida, Rhodotorula,* etc. can be demonstrated by the mere

Table 5. Concentrations of Phenylethyl Alcohol Required to Suppress Growth of Various Brewery Microorganisms*

Microorganism	Concentration; mg/ml of Medium				
	1	3	5	7	8
Gram-negative wort bacteria (strains A, X, Y)	+	−	−	−	−
Gram-positive coccus (strains 1,4)	+	+	+	−	−
Gram-positive coccus (strain 2)	+	+	+	−	−
Lactobacillus pastorianus	+	+	+	+	−
Lactobacillus sp.	+	+	+	−	−
Brewers yeast	+	−	−	−	−

*Data from Strandskov, F. B. *et al.*, 1953. Wallerstein Laboratories Communications 16, No. 52:31.

presence of a pellicle in a series of dilution tubes. On the other hand, the detection and enumeration of foreign yeasts, such as *Saccharomyces pastorianus, S. ellipsoideus, S. marxianus,* etc. whose physiology and morphology closely resemble *S. carlsbergensis* and *S. cerevisiae* is indeed rather difficult. Again, the effectiveness of antifungal antibiotics was investigated. While Actidione at 4 ppm inhibited the growth of *S. carlsbergensis* and *S. cerevisiae,* a variable response on growth inhibition of foreign or undesirable yeasts of the genus *Saccharomyces* and other related genera was observed (Table 6). Thus, *Saccharomyces marxianus* and some strains

Table 6. Effect of Actidione on the Growth of Various Wild Yeasts of the Genus *Saccharomyces** (Actidione 4 μg/ml in Wallerstein Laboratories Differential Medium)

Growth in Presence of Actidione	No Growth in Presence of Actidione
S. pastorianus ATCC # 2339	*S. pastorianus* (Hansen) NCTC
S. pastorianus ATCC # 4213	*S. pastorianus* "Winge" (Hansen)
S. marxianus	*S. carlsbergensis* var. *polymorphous*
	S. cerevisiae var. *ellipsoideus* var. *turbidans*
	S. diastaticus
	S. uvarum

*Unpublished paper.

of *S. pastorianus* are capable of growing in the presence of Actidione whereas other strains of *S. pastorianus* are not. Variants of *S. carlsbergensis* and *S. cerevisiae*, namely *polymorphous*, *ellipsoideus*, and *turbidans*, are also inhibited by Actidione. The effect of the antibiotic on the growth of yeast of other genera is shown in Table 7.

Table 7. Effect of Actidione on the Growth of Various Wild Yeasts*
(Actidione 4 μg/ml of Wallerstein Laboratories
Differential Medium)

Growth in Presence of Actidione	No Growth in Presence of Actidione
Torula cremoris	Saaz Yeast
Lactose fermenter	Brettanomyces Culture
Candida guillermondii	*Schizosaccharomyces octosporus*
	Lindner Wild Yeast

*Unpublished paper.

The availability of various carbon and nitrogen sources for the growth of various microorganisms, including yeasts, has, of course, been used as a classical taxonomic procedure. Sugars such as glucose, mannose, fructose, galactose, sucrose, maltose, lactose, raffinose and in some cases, inulin, have been used in yeast classification. In this connection, published data (Green and Stone, 1952) have shown that one of the fermentable sugars present in the brewers wort, namely maltotriose, can be utilized to differentiate between various culture and foreign yeasts (Table 8). A recent paper reporting on the utility of a series of carbohydrates in growth media as a means of detecting foreign yeasts in the presence of culture yeasts, indicated that this technique had promise in the separation of 10 species of foreign yeast from culture yeast (Wiles, 1954). Data from this study indicated that when cellobiose was used as the sole carbon source in an otherwise complete medium, members of *Candida, Hansenula, Kloeckera, Rhodotorula,* and *Torulopsis* genera were able to grow, whereas three species of *Saccharomyces* were not capable of utilizing cellobiose for growth (Table 9). Utilizing various carbon sources, 13 species of foreign yeast were isolated from English pitching yeast.

Another approach to the problem of rapid means of differentiating between various yeast genera and species has been adequacy of individual amino acids as sources of carbon for growth (Schultz *et al.*, 1949). Thus, the use of different amino acids in a growth medium differentiates between two closely related yeasts, *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* (Table 10).

Table 8. Differentiation Among Various Yeasts as Determined by Fermentability of Wort Sugar Maltotriose*

Maltotriose Fermented	Maltotriose Not Fermented
Brewers lager yeast (3 bios types)	Brewers lager yeast (1 bios type)
Brewers ale yeast (1 bios type)	Brewers ale yeast (1 bios type)
Saccharomyces pastorianus (1 strain)	*Saccharomyces pastorianus* (3 strains)
S. cerevisiae var. *turbidans*	*S. cerevisiae* var. *ellipsoideus*
S. thermantitorium	*S. cerevisiae* strain *illicis*
S. versitales	*S. carlsbergensis* var. *polymorphous*
Brettanomyces	*S. marxianus*
Lindner wild yeast	*S. uvarum*
Torulaspora fermentati	*Schizosaccharomyces octosporus*
	Saaz yeast

*Data from Green, S. R. and I. Stone, 1952. Wallerstein Laboratories Communications 15, No. 51:347.

Table 9. Cellobiose Assimilation of Various Yeast Species*

Yeast Species	Assimilation
Candida guillermondii	+
Hansenula anomala	+
Kloeckera apiculata	+
Rhodotorula mucilaginosa	+
Torulopsis famata	+
Pichia membranaefaciens	0
Saccharomyces carlsbergensis	0
Saccharomyces cerevisiae	0
Saccharomyces willianus	0

*Data from Wiles, A. E., 1954. Wallerstein Laboratories Communications 17, No. 59:259.

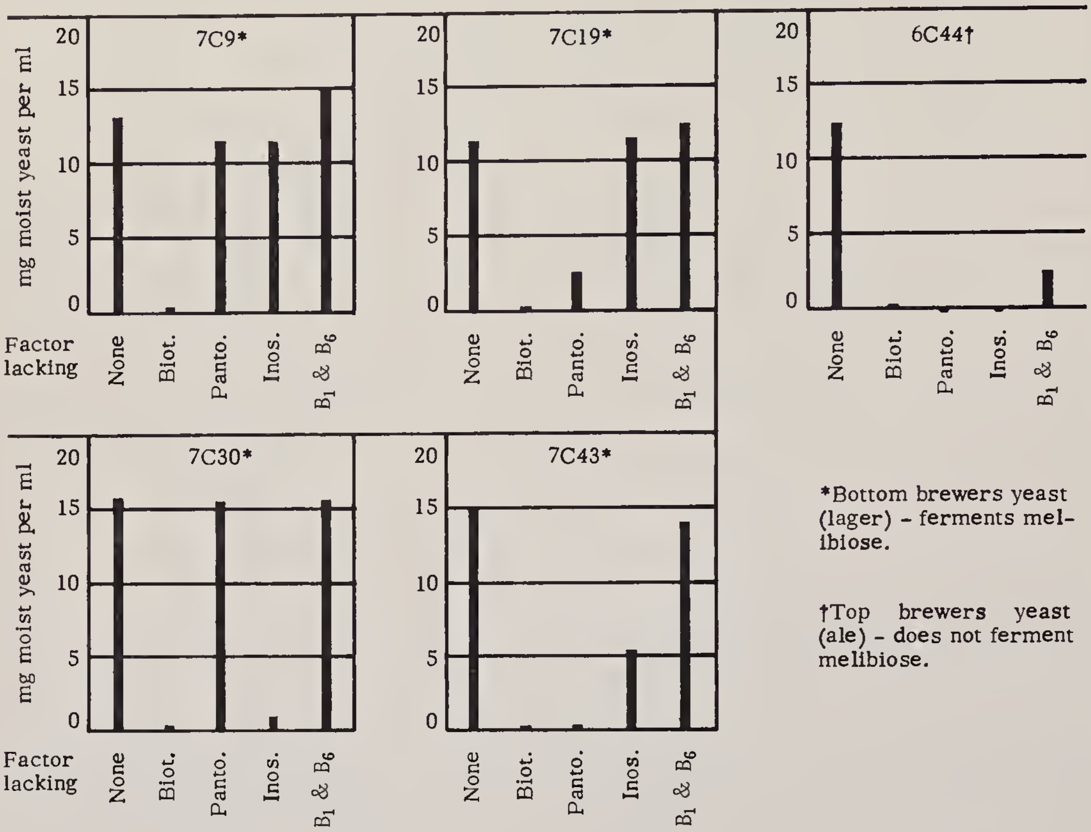
Table 10. Growth of Various Yeasts in a Medium Containing Amino Acids as the Sole Carbon Source *

Yeast	Amino Acid in Medium				
	Glutamic Acid	Proline	Aspartic Acid	Serine	Arginine
Saccharomyces cerevisiae	–	–	–	–	–
Zygosaccharomyces marxianus	+	–	–	–	–
Candida pseudotropicalis	+	+	–	–	–
Torula utilis	++	++	–	–	–
Candida guillermondii	++	+	++	–	?
Saccharomyces pastorianus	++	+	++	+	++

*Data from Schultz, A. S., *et al.*, 1949 Arch. Biochem. 22, 412.

Earlier in this review reference was made to the need for techniques which could detect the presence of new culture yeast strains or the shift in the proportion of known yeast strains in a brewers yeast inoculum. The prime requisite for this information is the fact that the adsorption of coloring material, foaming properties, taste, aroma, fullness, and the polish of the finished beer all depend, to some extent, on the specific yeast strain or strains used in beer fermentation.

One method which afforded a means of distinguishing various strains of ale and lager strains is based on the vitamin or "bios" growth requirements (Atkin, 1947). These requirements have been found to be reproducible and not to change with storage, serial transfer, or during successive fermentations in the brewery. Four different strains of brewers lager yeast have been recognized by means of the bios requirement procedure based on the random selection of 10 colony isolates (Fig. 1). Since the data from these tests were restricted to the vitamin B needs of solely 10 single colony isolates, it was quite possible that if some bios "types" were present in the inoculum in relatively small numbers, they could have been missed in the random selection of the colonies. Thus, for example, if a brewers yeast contained 10% of one bios type and 90% of another type, it would be expected statistically that about 10% of the time, on the average, no one set of 10 chosen colonies would represent the minority type. Similarly, analysis of growth requirements of the mass culture in various liquid vitamin media would mask the presence of some bios types. Shifts in the relative proportions of various bios types in a known mixed culture would be difficult to detect by the



*Bottom brewers yeast (lager) - ferments melibiose.

†Top brewers yeast (ale) - does not ferment melibiose.

Fig. 1

above techniques. Accordingly, a modified bios "typing" method involving the plating of the mass yeast culture was evolved (Green and Sullivan, 1959). The plating method was sufficiently sensitive to detect one yeast cell of one bios type in the presence of approximately 100,000 yeast cells of another bios type (Table 11). The relative proportion of bios types in commercial brewers yeasts and any deviation from this proportion during subsequent re-use in the brewery have been reported by means of the bios plating technique.

Table 11. Recovery of Various Bios Types in Laboratory-Prepared Mixtures Using the New Bios Typing Method

Mixture	Theoretical Proportions		% Recovery	
	Bios type C *	Bios type D†	Bios type C	Bios type D
1	99.999	0.001	99.9995	0.0005
2	99.0	1.0	99.0	1.0
3	90.0	10.0	89.0	11.0
4	10.0	90.0	13.0	87.0
5	0.1	99.9	0.09	99.1

*Bios type C is capable of synthesizing calcium pantothenate but not inositol.
†Bios type D is capable of synthesizing inositol but not calcium pantothenate.

Attempts have been made to classify brewers yeast strains by cell chain formation, extent of fermentation foam formation, aggregation at various acidic pH's, mutual precipitation by stock culture yeast, etc. (Hough, 1957). However, little correlation was obtained between brewery performance and classification based on the aforementioned techniques.

A test for flocculation ability of yeast which gave reproducible results with brewers yeast strains even after repeated subculture has been described (Kato and Nishikawa, 1957). However, comparison of the fermentation capacity and flocculative power of 12 yeasts with the quality and analysis of beers brewed with these yeasts showed no clear relationship (Rokita, 1956).

In addition to vitamin requirements, the assimilation of individual amino acids was found to be a valuable yeast strain characteristic; the most useful amino acids being norvaline, norleucine, and histidine (Streutker, 1959). Another study revealed that 4 strains of yeast could be distinguished according to their capacity of utilizing adenine and guanine from a complex wort medium (Harris and Parsons, 1958).

We have discussed, thus far, several means by which the fermentologist could detect and enumerate microbial contaminants in culture yeast and could note the

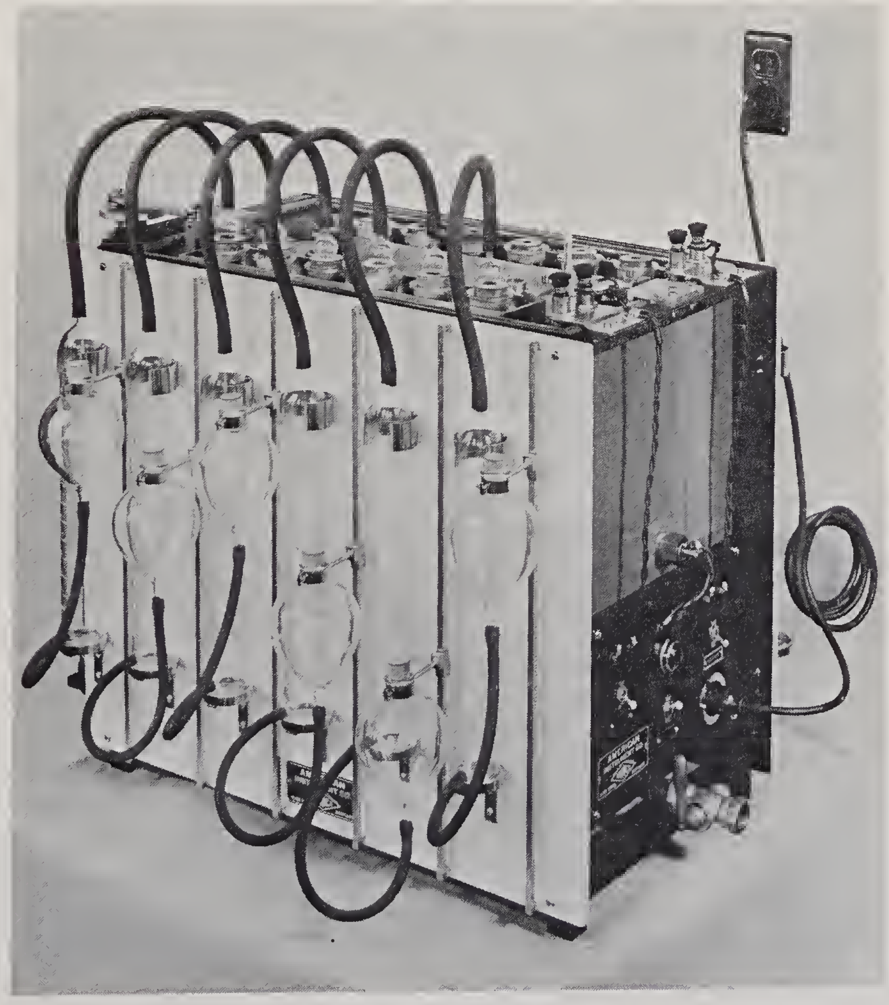


Fig. 2. Fermentometer.

entry of new culture yeast strains or shift in the relative proportion of yeast strains in a known mixed culture. However, still other microbiological data are required to provide for product uniformity. Information regarding the fermentation vigor of the yeast falls into this category.

As previously mentioned, brewers yeast may be re-used for as many as 100 fermentations. The cutoff point depends on extent of microbial contamination, strain characterization of the yeast, fermenting vigor of the yeast, etc. The use of a yeast which begins to exhibit sluggish fermentation mannerisms becomes undesirable since (a) it becomes "susceptible" to contamination, (b) the amount of yeast crop starts to decrease thus failing to provide sufficient yeast for subsequent fermentations, (c) a yeast "bite" may appear in the finished product, etc. While yeast vigor can be followed in the plant by means of specific gravity readings during fermentation, laboratory personnel can use the Fermentometer for this purpose (Fig. 2). The Fermentometer, a scale-up Warburg, measures the gas evolved from a semisynthetic medium. Three hours fermentation at 30C in the Fermentometer is equivalent to approximately 2 days' fermentation in the brewery.

In addition to assaying yeast vigor during continuous re-use, the Fermentometer has been used to note the effect of various physical and chemical agents on yeast fermentation (Green, unpublished data). Thus, effect of the acidity of acid washing* on yeast vigor can be determined. As indicated in Table 12, storage at excessive acidity reduces yeast fermentation capacity. Accordingly, washing at pH 3.5, which has been shown as satisfactory for inhibiting bacterial growth, has been utilized for the washing of brewers yeast (Green, unpublished data).

Table 12. Effect of pH During Washing* on the Fermenting Power of a Brewers Yeast

pH of slurry†	Fermenting Power ‡	Decrease (%)
5.2	1260	-
3.7	1120	11
3.2	955	24
2.8	700	44
2.6	465	63

*24-hr wash at 4-6C.
†pH adjusted by tartaric acid.
‡ml gas per g yeast solids, 30C fermentation for 3 hr.

A recent paper reported that data, obtained on measuring the fermentation "efficiencies" of 400 strains of *S. cerevisiae* and *S. carlsbergensis* indicated a statistically significant difference between the species (Thorne, 1958). Fermentation "efficiency" was regarded as an industrially significant property of a yeast strain. A complicating factor in this connection is the finding that variation in fermentation efficiency of single cell isolates increased during use in a brewery for several years (Thorne, 1957).

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*Yeast is "acid-washed" to reduce the reproductive capacity of bacterial contaminants.

SUMMARY

A review of various means of evaluating the strain purity and the fermentation vigor of brewers yeast was presented. Reference was made to the use of antibiotics for detecting and enumerating microbial contaminants in the presence of overwhelming numbers of brewers yeasts. The utility of sugars, nitrogen sources, B-complex vitamins, etc., for classifying brewers yeast was discussed. A laboratory method for determining the fermentation vigor of brewers yeast and the measurement of effect of physical agents thereon was presented.

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CONTRIBUTED PAPERS

moderated by J.M. Sharpley

MICROBIOLOGICAL SLUDGE IN JET AIRCRAFT FUEL

Alton E. Prince

Biomedical Laboratory, Wright Air Development Division, Air Research and Development Command
Wright-Patterson Air Force Base, Ohio

The problem of microbiological sludge in aircraft fuel first came to our attention during the summer of 1956 when samples of contaminated JP-4 fuel were brought to the laboratory from bulk storage tanks located at Davis-Monthan A.F.B., Arizona; Lincoln A.F.B., Nebraska; and Schilling A.F.B., Arkansas. This action was initiated because of refueling equipment malfunction in a KC-97 tanker aircraft and because of difficulties in the fuel systems of B-47 jet aircraft. Considerable effort was expended on these first samples to establish that microorganisms were a cause of sludge and not just present in the water bottoms of the storage tanks.

A preliminary review of available literature indicated that microorganisms, especially bacteria, could live in petroleum products. The JP-4 fuel is a low boiling kerosene with an initial boiling point of about 148 F and end point of 490 F. The material contains mostly aliphatic hydrocarbons with limited amounts of various cyclic compounds. Actually the jet fuels are closely controlled by the requirements of Specification MIL-J-5624E.

Initial experiments (Bakamauskas, 1948) were designed to accomplish the following:

1. Establish the presence of microorganisms in the sludge.
2. Isolate the bacteria in pure culture.
3. Establish whether or not the bacteria could cause sludge.
4. Determine whether or not approved corrosion and gum inhibitors, normally added to the fuel, were bacteriostatic agents.
5. Investigate selected water-soluble, fuel-insoluble chemical compounds as bacteriostatic sludge inhibitors.

It was relatively easy to establish the presence of bacteria in the sludge and about 70 separate isolates were obtained. The standard media used were Soil Extract, Starch, Levine EMB, Potato Dextrose, Glucose, and Thioglycollate solid agars. The last was used with the Brewer plates for anaerobic forms. No filamentous fungi or anaerobic bacteria were found in these first samples.

Seventeen of the original isolates were selected on the basis of gross apparent differences such as growth rate, color of medium, color of colonies, and relative size of individual cells. The most important characteristic was considered to be growth rate. Some of these isolates grew very fast, overnight being too long a time for the isolation of cells from single colonies, while others which were very slow growers were not utilized for additional experiments. Some forms produced very stable emulsions of the fuel and water, while other forms produced gumlike residues which settled into the water phase. Some colored the solid media bluish, others greenish, and others produced water-soluble pigments in various shades of red. Considerable effort was expended attempting to identify these isolates but often their response to various identifying culture techniques was anything but diagnostic. Genera definitely identified were *Pseudomonas, Aerobacter,* and *Bacterium.* Later samples have yielded many other forms including a Gram-negative diplococcus, spirochaetes, and a *Clostridium.* Sulfate-reducing forms have been identified subsequently from sludge samples collected in Spain, Puerto Rico, and from Chenault Air Force Base, Alabama. They are known to be present in North African bases from an excellent, but unpublished, study by E. J. Robles, who was stationed at Nouasseur, Morocco.

The anaerobic sulfate-reducing bacteria are considered to be of minor importance as sludge formers because they have been found in so few of the large number of samples received for examination. It is believed that most storage tanks are utilized enough so that filling and emptying operations produce enough aeration to keep them under control. It is possible, however, that the method of sampling has not been conducive to their survival. In aircraft it is believed that the amount of aeration certainly could be a limiting factor for their survival.

Attempts to control the growth of the contaminating bacteria in the first samples yielded some interesting results. Fuel-soluble inhibitors were considered at first because approved corrosion and gum inhibitor additives were for the most part substituted tertiary phenols. One compound in particular, 2,6-ditertiary-butyl-phenol, was thought to be a potential inhibitor at 5% by weight in the fuel over the Bushnell-Haas medium. It was only a matter of a few days when most of the isolates were growing just as prolifically, or more so, with this and similar inhibitors as they were without the chemical. It soon became evident that these organisms were capable of adapting their physiology to very adverse conditions. It was believed at that time that if fuel-soluble inhibitors could be found, they would have to be used at such high concentrations that potential danger of adverse effects on aircraft and engine parts and the high cost would make them prohibitive. Because the inside of the wings of jet aircraft are mostly fuel tanks, any material that might cause corrosion to aluminum could not be considered. These facts turned attention to potential water-soluble, fuel-insoluble microbicidal compounds.

Since JP-4 fuel at 70F contains approximately 70 ppm water in solution, it was essential to look for compounds that could not cause adverse affects to fuel or aircraft. This meant that the usual compounds containing metals such as copper or mercury could not be used and that compounds containing halogens probably would not be acceptable because these chemicals might be corrosive or leave harmful deposits.

A review of the literature showed that a patent (Harris and Strawinski, 1954) had been issued indicating that either sodium or potassium tetraborate might be an effective control agent. Laboratory experiments confirmed that the bacteria on hand could be kept under control at 1.5% by weight of water.

The next step was to store jet fuel over water containing a concentrated, about 6%, solution of sodium tetraborate for about 30 days, then run a jet engine on this fuel for 100 hr. This is required of any additive and was accomplished by the Propulsion Laboratory at Wright Air Development Division. Results of this evaluation were acceptable and a field trial was considered.

Sometime later a severe sludging problem was reported at Chenault A.F.B., Louisiana. The recommendation was given that the bulk storage tanks be cleaned and when the tanks were put back in use that 2% by weight commercial grade sodium tetraborate be added to the water that collects in the bottom of the large storage tanks. Problems developed because the bottoms of the tanks are not regular making it difficult to estimate the amount of water present. In addition, one inch of water in one of the very large tanks represents about 800 gallons. It was difficult for field personnel to add the amount of chemical required and even more difficult to maintain the required concentration. In some tanks the bacterial population was kept down, in others the bacteria seemed to multiply very fast even though the sodium tetraborate was at least at the 2%-by-weight concentration. Present recommendations are to keep the tanks as free of water as possible and keep them clean. The search for acceptable water-soluble, fuel-insoluble inhibitors is continuing with a contract at Gulf Research and Development Company in Pittsburgh. This is a cooperative effort between the Materials Central and the Biomedical Laboratory at Wright Air Development Division.

If only bacteria were the cause of sludge the problem would be much easier to solve. A great many samples of aircraft fuels have been received from operating aircraft and it should be mentioned that microorganisms have been isolated also from the high octane aviation gasoline. It has been concluded that airborn spores or mycelial fragments of the microorganisms find their way into the bulk storage tanks with rain water that leaks in around the floating roof of the tank. In addition to bacteria several different forms of filamentous fungi have been isolated. Many do not produce spores. Genera easily recognized include *Penicillium, Aspergillus, Spicaria,* and *Helminthosporium.*

Pictures have been taken of some of these and have been included. Figure 1 is of what I have called a *Spicaria,* and the other figures are of different stages of the fungus that I have called a *Cladosporium.* Figure 2 shows a general view, Fig. 3 is a close-up of the mycelium and spores. Figure 4 is an enlarged single conidiophore with spores. This fungus is of particular importance because additional experimentation has been done with it. The mycelium is dark and when grown in a tube between Bushnell-Haas medium and JP-4 fuel, it grows up into the fuel layer forming a very dense mat. It seemed possible that this microorganism might be utilizing the fuel more than some of the others. A good report showing that microorganisms can utilize and convert hydrocarbons is given by Kallio (1959). It was decided to try to measure the effects of this fungus and some bacteria on the JP-4 fuel, utilizing gas chromatographic techniques. The results of these preliminary investigations are shown in the last three illustrations. Figure 5 is a graph produced by the recorder of a gas chromatography apparatus. The line to the right shows the constituents of the regular JP-4 fuel, and the line to the left shows that several of the initial peaks are missing. This indicates that the microorganisms are utilizing, in some way, the light fractions of the fuel. The next two illustrations show this even more clearly. Figure 6 shows the line of the original JP-4 fuel, and Fig. 7 shows that line after exposure of the fuel to the fungus for 5 weeks—all of the initial peaks have disappeared. Very similar

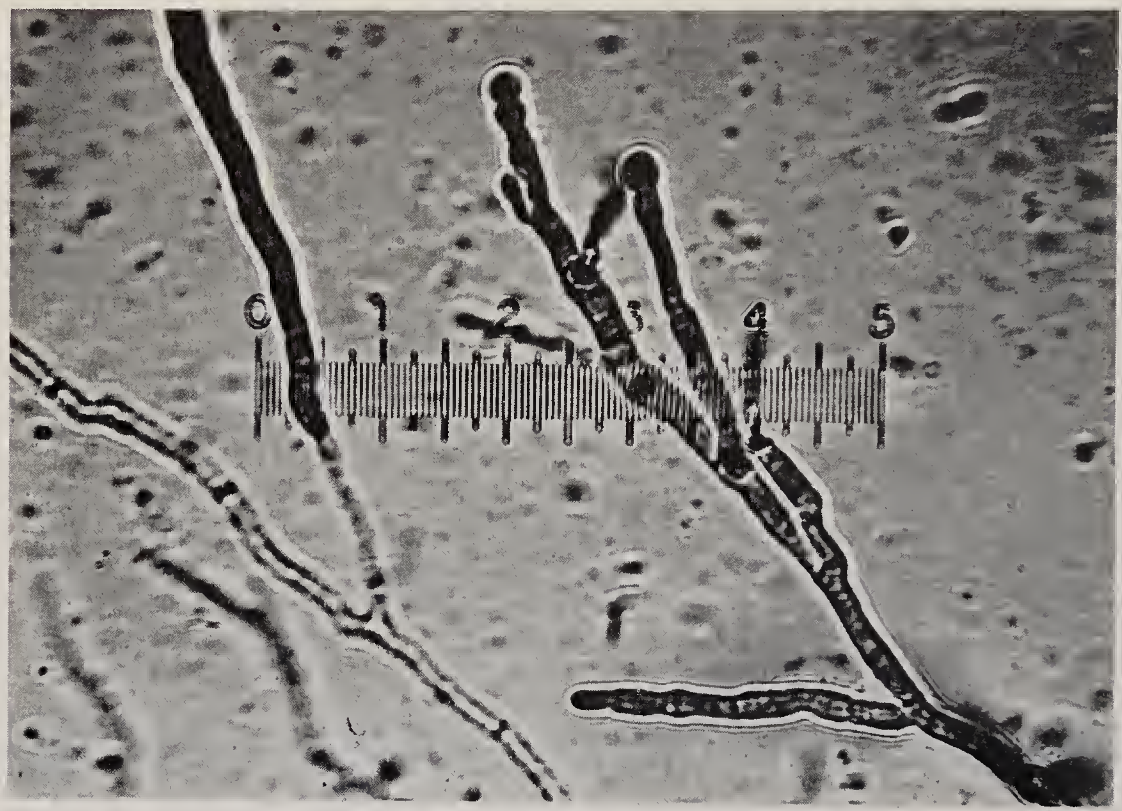

Fig. 1. Hyphae and young conidiophores with spores of *Spicaria*.

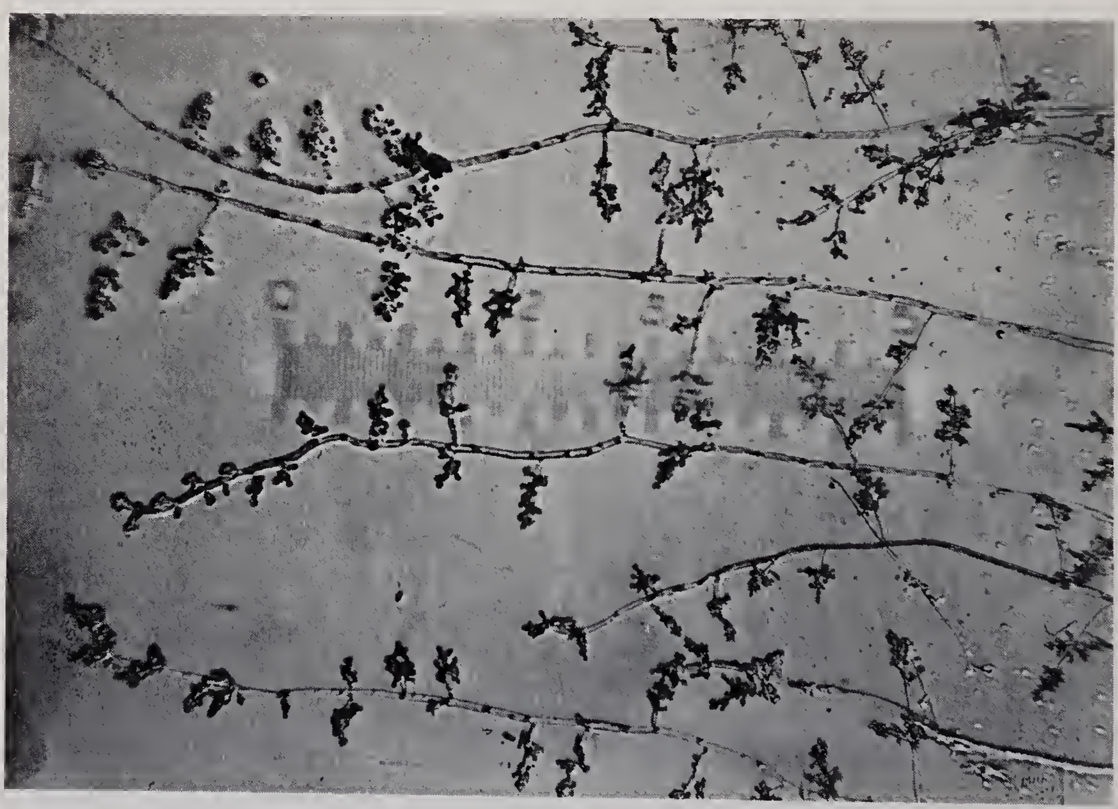

Fig. 2. *Cladosporium*. Gross view of mycelium, conidiophores, and spores.

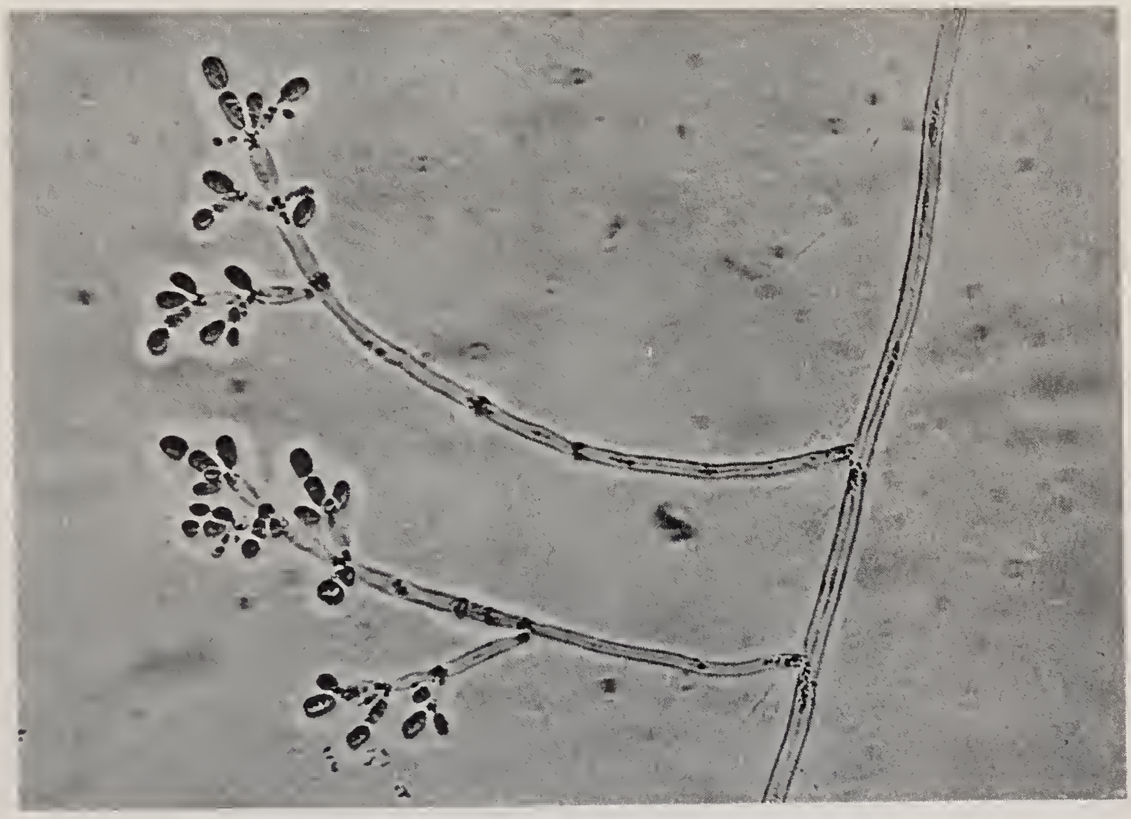

Fig. 3. *Cladosporium* . Young conidiophores and spores.

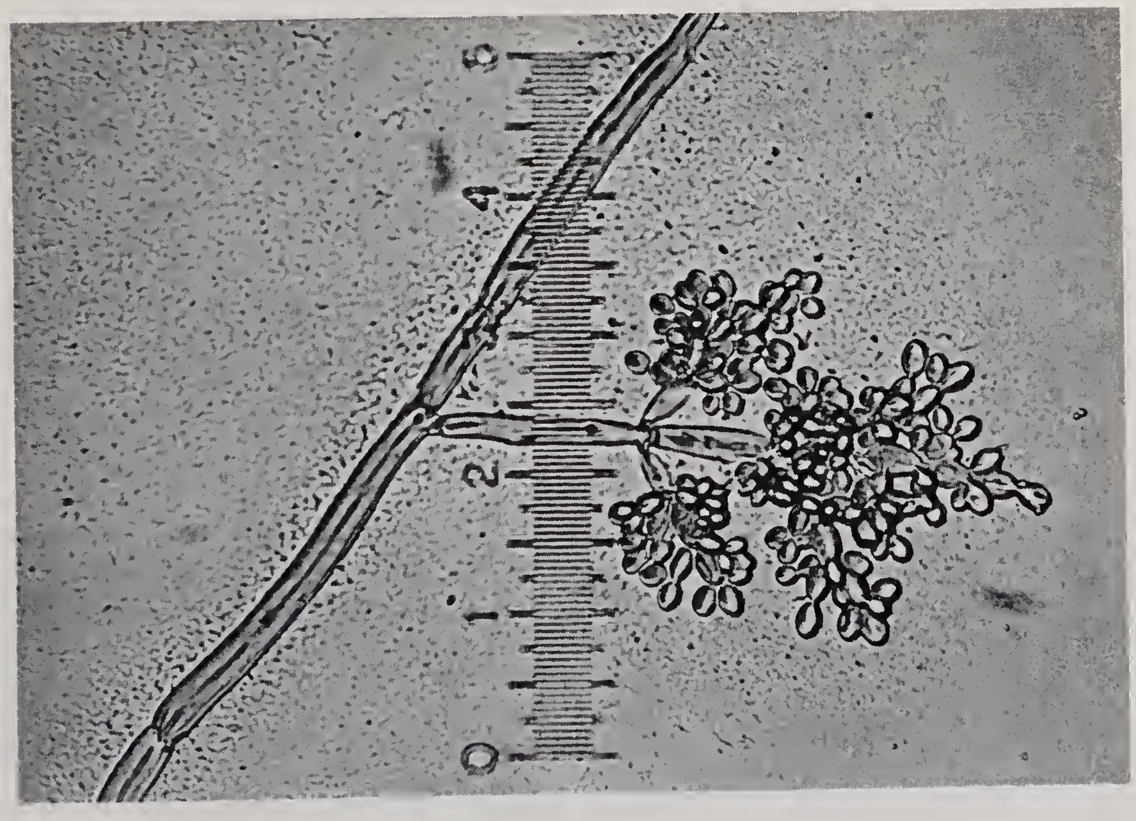

Fig. 4. *Cladosporium* . Mature conidiophore and spores.

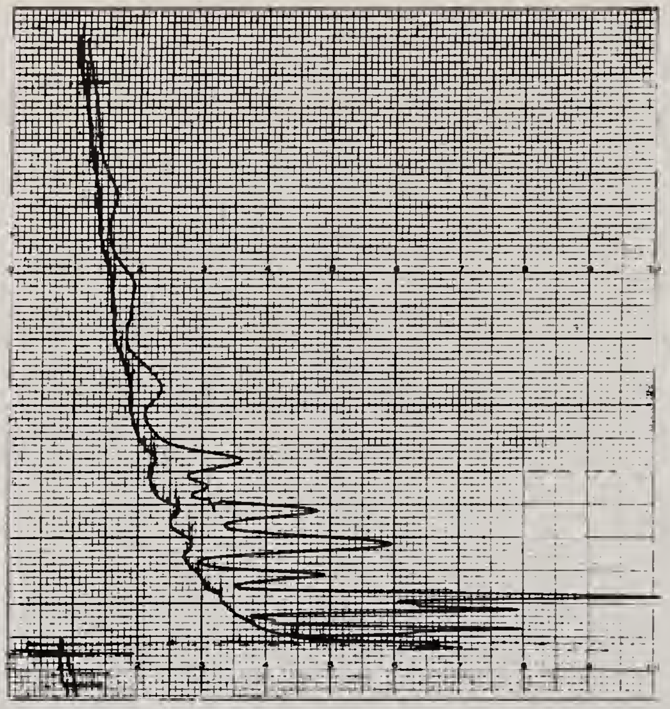

Fig. 5 Gas chromatograph of JP-4 fuel and JP-4 fuel inoculated with *Cladosporium*. The line to the right represents the fractions present in sterile JP-4 fuel; the line to the left represents the fractions in JP-4 fuel inoculated with *Cladosporium* and incubated 5 weeks.

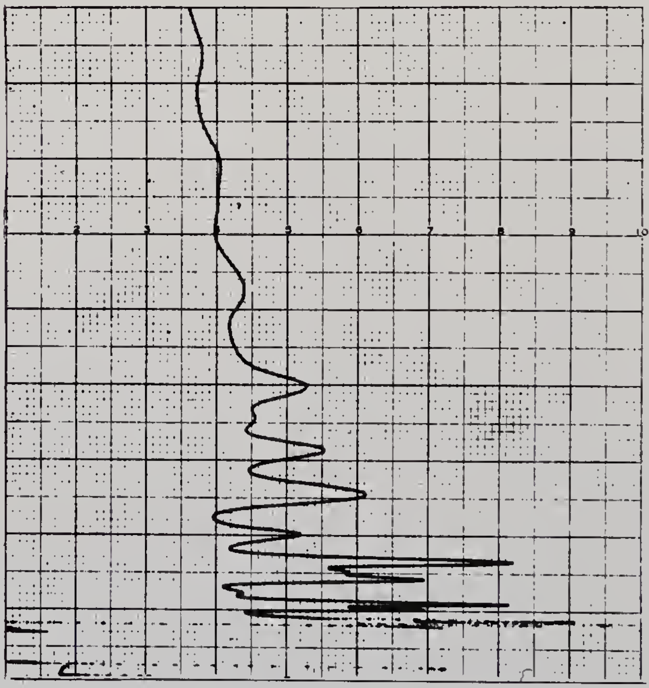

Fig. 6. Gas chromatograph of sterile JP-4 fuel.

results have been obtained with bacteria. The specific compounds represented by the peaks shown on the graphs are not known, at the present time, but these results do show that these microorganisms can and do alter the components of the fuel. Additional work is planned to determine the extent of these changes and the effects on the fuel.*

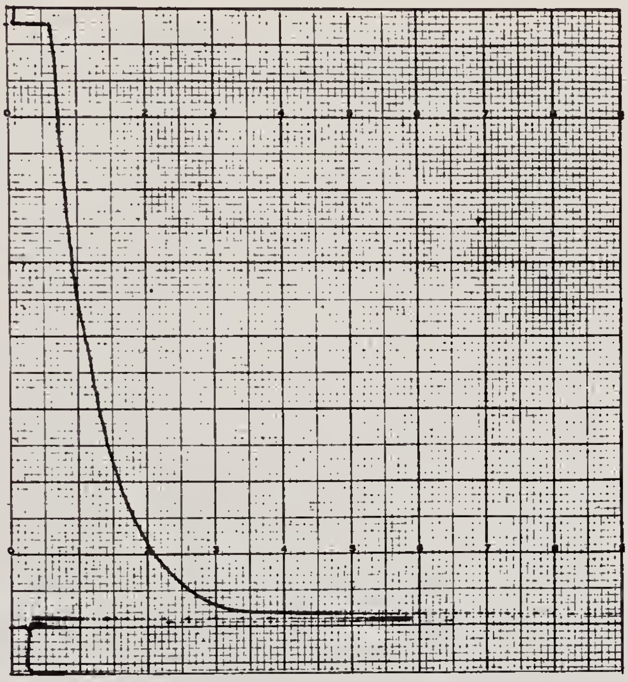

Fig. 7 Gas chromatograph of JP-4 fuel inoculated with *Cladosporium* and incubated 5 weeks.

ACKNOWLEDGMENTS

Acknowledgment is due my former colleague, Mr. Sam Bakamauskas, Materials Central, WADD, for his efforts in the original investigations and to Mr. Charles Underwood for excellent photography needed to obtain the pictures of the living microorganisms. Special credit is due Miss Marilyn George and Mrs. Mildred Pinkerton for their efforts in developing ways to analyze the fuels utilizing the gas chromatograph. It is believed that this particular kind of analysis has not been accomplished previously.

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*Subsequent Warburg experiments by Miss George show that carbon dioxide is a product of metabolism, indicating complete breakdown of some fuel fractions.

MISO. II. FERMENTATION

K. Shibasaki* and C. W. Hesseltine †

Northern Regional Research Laboratory,‡ Peoria, Illinois

For centuries miso, a food prepared from fermented whole soybeans, has been widely used in Japan as a base for a hot breakfast soup. Several different types of miso are manufactured which vary in color and composition of the fermented ingredients. Primarily, miso is made from cooked soybeans to which a koji (molded rice) and salt have been added. The mixture is inoculated with miso, and enzyme action, fermentation, and ripening take place. Conditions under which the soybeans are steamed, the salt concentration, the amount of koji used, and the flavoring in the ingredients employed, all affect the type of final product. For example, Sendai miso has a reddish-brown color, and the soybeans in it are cooked 2 hr at 10 psi and 30 min at 5 psi. Shinshu miso, which is yellowish-brown, is made from soybeans cooked for 1 hr at 7 psi. Typically, koji for all miso is made as follows: steamed rice is inoculated with the spores of *Aspergillus oryzae* and incubated for approximately 50 hr at about 27-28 C until the rice is completely covered with mycelium but before sporulation occurs.

In a search for ways to increase the use of U.S. soybeans in traditional Japanese foods, better processing and fermentation methods have been studied. Because U.S. and Oriental soybeans differ, current Japanese food-processing methods do not produce the best possible miso from U.S. beans. Two approaches might be used to encourage the increased use of U.S. soybeans in miso: (1) To supply a selected variety or varieties of U.S. soybeans comparable in food uses to Japanese beans, or (2) to modify Japanese food processing methods so that soybeans from the U.S. could be used to give equally good miso, similar to that produced from Japanese soybeans. Under present methods of marketing and exporting soybeans, the solution of Japanese problems by selecting a particular variety for export might be difficult. Such a procedure would certainly make a substantial increase in the export price.

MATERIALS AND METHODS

The same varieties of soybeans were used in this work as were described in the first publication of this series (Shibasaki and Hesseltine, 1960). The beans were from the 1957 and 1958 crops.

The kojis used in the experiments were prepared from a Japanese commercial tane-koji (koji-starter). The inoculum is in the form of dry spores of

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*Present address: Faculty of the College of Agriculture, Tohoku University, Sendai, Japan.

†In charge of ARS Culture Collection at Northern Regional Research Laboratory.

‡This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

Aspergillus oryzae and may represent more than one strain of *A. oryzae.* The koji is prepared from American polished short rice which has been soaked in water for 17 hr at approximately 25 C, cooked with steam for 70 min, cooled and inoculated with the tane-koji. The inoculated rice is spread in wooden trays with the inside dimensions of 23 × 40 cm with a depth of 5 cm. The trays must be soaked in water prior to filling with rice, so that the rice, even next to the wood, will not dry out with the consequent inhibition of mold growth. The rice must be spread to a depth of 3 cm in order that the mold will grow about every kernel of rice. The temperature in the rice during fermentation must not exceed 43 C. The trays are incubated at 28 C. Typically, the temperature of the rice should rise to slightly above 40 C on the second day in order to form maximum amounts of proteolytic enzymes. The entire period of incubation amounts to approximately 50 hr. When a number of trays are used, it is important to allow free movement of air between them over the molding rice. Under no circumstances should the mold development progress to sporulation or pigmentation of the mycelium. If the mold sporulates, a moldy flavor will occur and there is danger of contamination of other microbiological cultures in the same laboratory. Three kg of rice makes approximately 3.85 kg of koji.

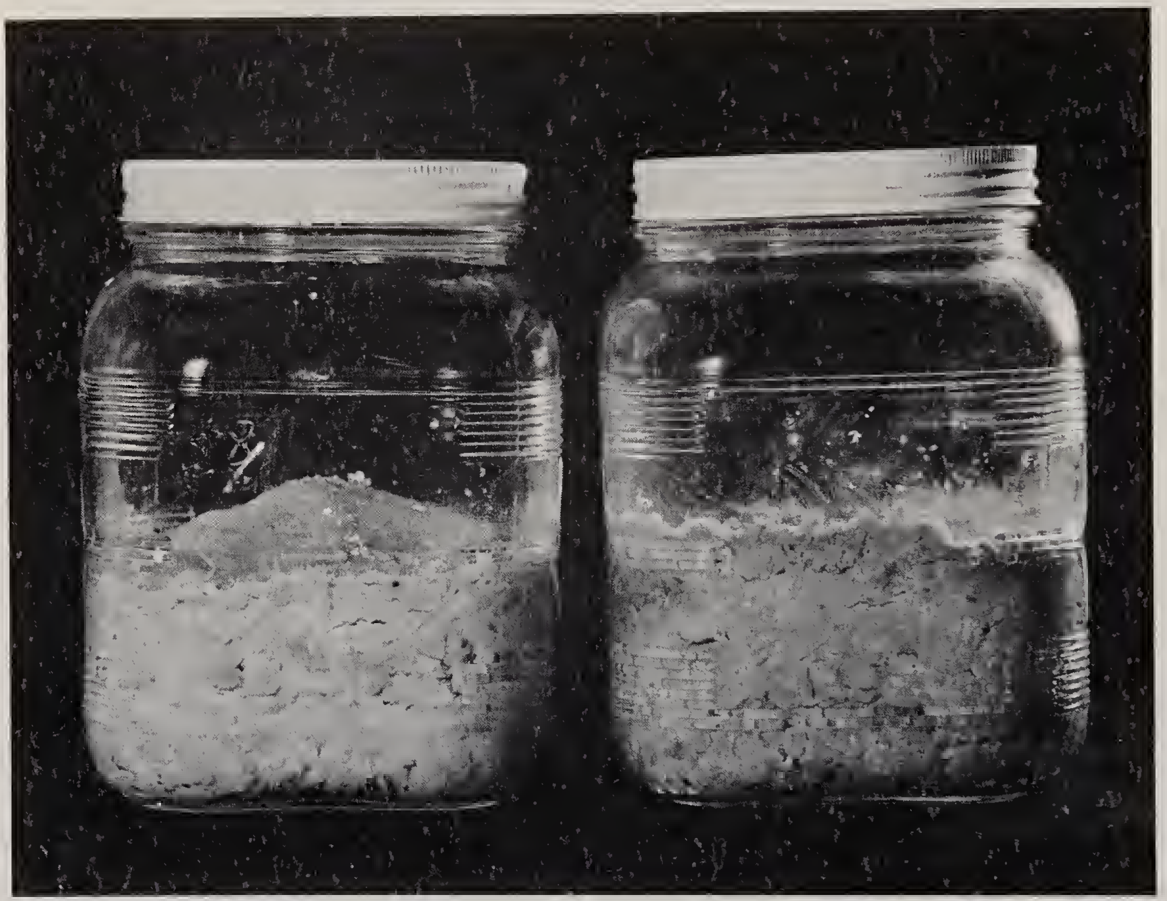

Fig. 1. Glass jars containing small amounts of fermenting miso.

The fermentations were carried out in incubators at about 35 C. For small-scale fermentations, glass jars of 3,000 ml capacity with metal screwtype lids were found to be quite satisfactory. These jars (Fig. 1) had mouths of 12 cm in diameter and were 21 cm in height. It is necessary to have a wide-mouthed jar in order to place the ingredients in the jar, to pack the substrate tight prior to fermentation, and to remove the finished product. The jars were filled to a depth of about 8 cm and the surface covered with Parafilm.* During the fermentation and aging the screw caps were kept on tightly. For larger fermentations, stainless steel containers of 32-kg capacity were used; again the surface of the substrate during a large fermentation and aging was covered with Parafilm and a weight placed on top of it. No special precautions were observed, except that fermentation vessels were kept covered to prevent entrance of contaminating molds. However, the rice and soybeans were both cooked and their natural flora destroyed. The salt was not sterilized and special precautions were not observed in mixing the media together or in inoculating.

RESULTS

Eleven varieties or types of soybeans were fermented to prepare two types of miso. Each kind of soybean was soaked in water for 16.5 hr at 25 C. For Sendai miso (miso with a reddish-brown color) soybeans were cooked for 2 hr at 10 psi and then further cooked for 30 min at 5 psi to give the desired dark color. For Shinshu miso (yellow-brown color) soybeans were cooked for 1 hr at 7 psi to prevent the beans from becoming too dark in color. After being soaked in water and cooked with steam, the soybeans absorbed water as shown in Table 1. All American varieties absorbed over twice their weight in water.

The beans soaked and cooked as described in Table 1 were then blended with mold rice, and salt was added according to the proportions given in Table 2.

The proportions of beans and rice were based on dry weight. The salt represents about 11.6% as based on the wet weight of the soybeans and rice. The inoculum was miso of good quality brought from Japan and was dissolved in the water prior to inoculation of the substrate. The soybeans, mold rice, salt, inoculum, and water were mixed together and lightly mashed with a sausage grinder. This mixture was then tightly packed by hand in the wide-mouthed glass containers where the soybeans underwent fermentation for 3 to 5 months, at about 35 C. Throughout the fermentation period the surface of the substrate was covered with parafilm with the lids of the containers kept tightly closed. No large amounts of gas accumulated, although the odor given off had a pleasant yeast-like smell. In some cases yeast growth could be seen at the top of the substrate beneath the parafilm. During the fermentation there was a gradual darkening of the miso. At the end of the fermentation, the jars were allowed to age for about 4 weeks at room temperature.

Since the making of miso using whole U.S. soybeans could be satisfactorily duplicated in the laboratory, another approach was taken to find a better processing method. Accordingly, we investigated the use of full fat grits (soybeans from which the seedcoats are removed and cracked into several pieces).

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*The mention of products does not imply endorsement or recommendation by the Department of Agriculture over other products of a similar nature not mentioned.

Table 1. Weight of Soybeans after Soaking and Steaming

Variety or kind	Weight (g)					
	Initial (1)	After soaking (2)	Increase after soaking (2)-(1)	After steaming (3)	Increase after soaking and steaming (3)-(1)	Decrease after steaming (2)-(3)
Shinshu Miso						
1. Chippewa	500	1144	644	1034	534	110
2. Adams	500	1157	657	997	497	160
3. Lincoln	500	1170	670	1030	530	140
4. Clark	500	1169	669	1038	538	131
5. Harosoy	500	1150	650	1001	501	149
6. Hawkeye	500	1171	671	1051	551	120
7. Hokkaido-Akita	500	1239	739	1103	603	136
8. Iwate	500	1254	754	1102	602	152
9. Miyagi	500	966	466	860	360	106
10. Nagano	500	1147	647	1001	501	146
11. Chinese	500	1189	689	1077	577	112
Sendai Miso						
1. Chippewa	500	1161	661	1035	535	126
2. Adams	500	1208	708	1022	522	186
3. Lincoln	500	1194	694	1036	536	158
4. Clark	500	1186	686	1062	562	124
5. Harosoy	500	1180	680	1043	543	137
6. Hawkeye	500	1219	719	1065	565	154
7. Hokkaido-Akita	500	1232	732	1113	613	119
8. Iwate	500	1244	744	1100	600	144
9. Miyagi	500	967	467	889	389	78
10. Nagano	500	1160	660	999	499	161
11. Chinese	500	983	483	869	369	114

Table 2. The Proportions of Soybeans, Rice, and Salt in Shinshu and Sendai Miso

Miso	Soybean (g)	Rice (g)	Salt (g)	Water* (ml)
Shinshu	500	300	200	100
Sendai	500	250†	200	100

*Eight grams of good miso is added in water (as starter).
†Commonly 200 g of rice is used in Japan.

Soybeans (variety Hawkeye) were crushed by a roller-type crusher into grits and screened through a sieve that had $^{11}/_{64}$ in. round holes and a 4 × 22 in. slot. The yield from 2,270 g of beans was 1,873 g of grits, 265 g of hulls, 96 g of germ and 64 g of overs. The yield of grits, which had a moisture content of 11%, was 83%. Only grits were studied in the remaining fermentations. In order to get the grits to absorb water, they were sprayed with a calculated amount of water and allowed to stand for absorption, or were soaked for a short time in water.

Spray Method of Adding Water

Two sizes of grits were tested. Beans crushed into 5 to 8 pieces were considered to be fine grits while coarse grits were beans crushed into 2 to 4 pieces. These grits were sprayed with water in the proportions shown in Table 3. After the addition of water, the grits were allowed to stand for 5 hr at 27 C and occasionally mixed to obtain uniform moisture distribution. The grits were then cooked for 1 hr at 7 psi. The results are shown in Table 3. Sixty g of water to 40 g of grits was adequate for both coarse and fine grits. Grits which were sprayed with less water were hard and did not cook completely.

Table 3. Characteristics of Coarse and Fine Soybean Grits Following Soaking and Cooking*

	Coarse			Fine		
Grits (g)	60	50	40	60	50	40
Water (g)	40	50	60	40	50	60
Total (g)	100	100	100	100	100	100
Weight after cooking (g)	105.5	102.9	88.9	103.9	100.2	86.7
Hardness	Hard	Medium hard	Soft	Hard	Medium hard	Soft
Color	Orange-brownish yellow	Yellow	Whitish yellow	Orange-brownish yellow	Yellow	Whitish yellow

*Soaked in water for 5 hr at 26.6C and cooked at 7 psi for 1 hr.

A study was next made to determine the amount of time necessary for grits to absorb water. Lots of 40 g of grits sprayed with 60 g of water were allowed to stand for 1, 3 and 5 hr after which they were cooked for 1 hr at 7 psi. There was no marked difference in hardness, elasticity and color among the grits after they were cooked. After being cooked, the grits weighed 85, 84 and 83 g, respectively. Consequently, a soaking time of 1 hr for grits was adequate.

Next, the time required for cooking was studied. Lots of 40 g of grits were soaked with 60 g of water for 1.5 hr and cooked for 20, 40, 60 and 90 min at 5 psi. The weights of cooked grits were 92.0, 92.2, 91.4 and 94.0 g, respectively. The grits cooked for 20 min were not so elastic and were somewhat hard. Grits cooked for 40 and 60 min were well cooked and had no marked differences in color, hardness, and elasticity. Grits cooked for 90 min were overcooked and had darkened slightly.

Soaking for a Short Time in Water

When a calculated amount of water, sufficient to cover the grits initially, was added, they absorbed water uniformly. However, since the water was quickly absorbed, there was soon no water left in the top layer of grits. Consequently, the grits did not absorb water uniformly. On a laboratory scale the grits could be mixed easily and completely, but on an industrial scale mixing might be difficult. For this reason, a method of soaking a short time was investigated as follows: The grits were soaked in water, drained and allowed to stand for a few hours to allow the water to penetrate completely. Fifty g each of whole beans, coarse grits and fine grits were weighed and then soaked in water for varying periods of time at 25 C. The absorption of water and increase in weights are shown in Table 4.

Table 4. Rate of Water Absorption of Hawkeye Soybeans

Soaking time (min)	Increase in weight (g)		
	Whole beans 50 g	Coarse grits 50 g	Fine grits 50 g
20	56.5	86.0	102.0
40	61.0	95.5	111.0
60	63.0	102.0	115.0
80	65.0	107.0	117.0
140	74.0	124.0	122.0
200	81.0	124.0	124.0
260	86.0	126.0	126.0

Obviously whole beans (Hawkeye) absorbed water very slowly, but both sizes of grits absorbed water very easily. It may be sufficient to soak fine grits in water for 80-100 min, but coarse grits need about 120 min.

In spite of the fact that grits absorbed water very easily, they should be allowed to stand a few hours after soaking and before cooking. In one experiment 40 g of grits were soaked in 120 ml of water at 25 C for 1.5 hr, were allowed to stand for 1, 3, and 5 hr respectively, and then cooked for 1 hr at 7 psi. Since all had the same elasticity, softness, and color, under these conditions it was sufficient to allow the grits to stand for 1 hr. For example, in one experiment 40 g of grits were soaked in 120 ml of water for 1.5 hr and then were allowed to stand at various intervals before cooking for 1 hr at 7 psi. The results were as follows:

97 g after standing 1 hr before cooking; 81 g after cooking.
95 g after standing 3 hr before cooking; 84 g after cooking.
94.5 g after standing 5 hr before cooking; 82 g after cooking.

Fermentation of Grits

The fermentation of grits is illustrated in the following three examples.

Fermentation 1. Hawkeye soybeans were cracked into grits by a roller-type crusher as described above. The grits (4,994 g) were soaked in 17,500 ml of tap water for 100 min and then drained of excess water to give 11,849 g of wet grits. The grits were then cooked with steam for 1 hr at 7 psi. The weight of the grits was then 10,917 g and had a moisture content of 59.3%. They were soft, uniform in texture, yellow-colored, and attractive in appearance. One thousand g of these grits were then mixed with 370 g of koji, 200 g of sodium chloride and 70 ml of tap water, and inoculated with 30 g of good miso from a previous fermentation. The mixture was then packed tightly into the glass jars, the lids were placed on the jars tightly, and the fermentation was allowed to go for 7 days at 28 C and then the jars were kept for 2 months at 35 C. No aeration was supplied. The fermentation was completed at this time and the miso allowed to age for 2 weeks at room temperature and was then ground into a thick paste. The product had an excellent appearance, flavor, taste and odor. This type of miso is called yellow miso (Shinshu miso).

Fermentation 2. Eight hundred grams of grits, soaked and cooked like those in Fermentation 1, were mixed with 500 g of mold rice, 130 g of sodium chloride and 50 ml of tap water. The mixture was then seeded with 30 g of inoculum taken from a previous, good miso fermentation. The thoroughly mixed material was fermented in a jar, as described before, for 1 week at 28 C. At the end of this time, the temperature was changed to 35 C and the fermentation allowed to continue for 3 weeks. The fermentation was now complete and the miso was allowed to age for 1 week at room temperature. The miso was then ground into a paste. This white miso had excellent appearance, flavor, odor, and a light color.

Fermentation 3. A pilot-plant fermentation was made with grits from Hawkeye soybeans processed like those in the first fermentation. Five kg of soybean grits were sprayed with 7.5 L of tap water and allowed to stand for 2.5 hr with occasional stirring. This batch was cooked with steam for 1 hr at 5 psi. The weight after cooking amounted to 10.407 kg with a moisture content of 57.2%. The grits were very soft, uniform in appearance, and were a pleasing yellow. To the cooked grits were added about 4.0 kg of mold rice, 2.0 kg of sodium chloride and 0.75 kg of water. The mixed substrate was inoculated with 0.25 kg of miso from an earlier good fermentation. The material was then placed in a large stainless steel tub, covered with Parafilm, allowed to stand for 7 days at 28 C, and finally removed for 2 months to an incubator in which the temperature was held at 35 C. At the end of 2 months the fermentation was complete and the product was aged at room temperature for 2 weeks. The finished product yielded approximately 17 kg of yellow miso which had excellent yellow color, flavor and odor. A flow sheet for the above fermentation is shown in Fig. 2.

Difficulties are always encountered in evaluating a food product of this nature, since its acceptability is dependent on color, odor, and taste; these factors cannot be readily translated into precise data. However, we had some of the material tested for its amino acid content by microbiological assays and found the following in terms of $\mu g/g$ of final product. Fermentation 2: lysine

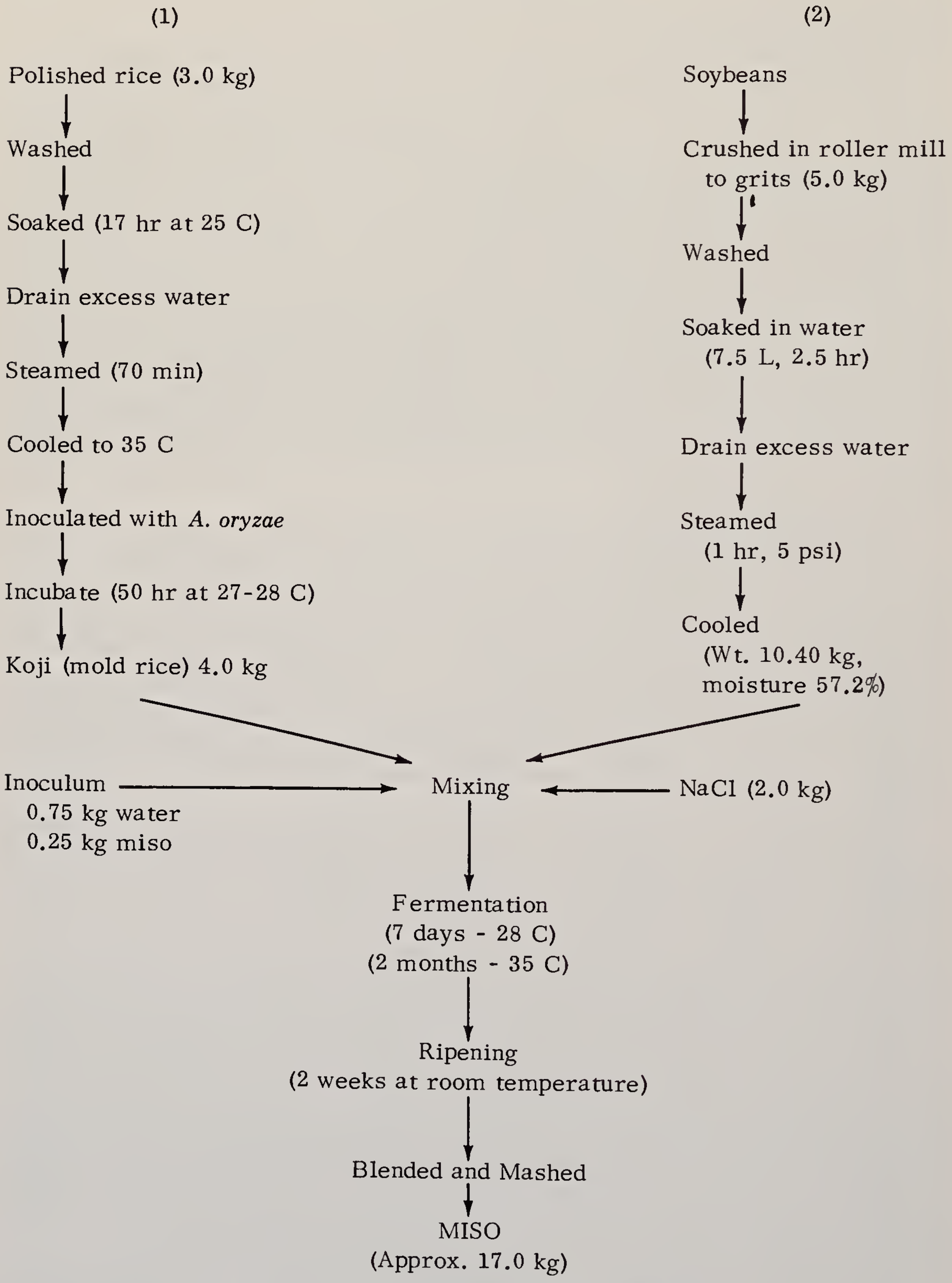
(1)
(2)
Polished rice (3.0 kg)
Washed
Soaked (17 hr at 25 C)
Drain excess water
Steamed (70 min)
Cooled to 35 C
Inoculated with *A. oryzae*
Incubate (50 hr at 27-28 C)
Koji (mold rice) 4.0 kg
Soybeans
Crushed in roller mill to grits (5.0 kg)
Washed
Soaked in water (7.5 L, 2.5 hr)
Drain excess water
Steamed (1 hr, 5 psi)
Cooled (Wt. 10.40 kg, moisture 57.2%)
Inoculum 0.75 kg water 0.25 kg miso
Mixing
NaCl (2.0 kg)
Fermentation (7 days - 28 C) (2 months - 35 C)
Ripening (2 weeks at room temperature)
Blended and Mashed
MISO (Approx. 17.0 kg)

Fig. 2. Flow sheet for the production of miso from grits.

12,000, glutamic acid 37,000, and methionine 2,500. Fermentation 3: lysine 12,500, glutamic acid 43,800, and methionine 2,200. A vitamin B_{12} assay was made on the two samples; there was less than 0.02 μg/g.

DISCUSSION

Recently, the use of large amounts of U.S. soybeans in Japan has caused difficulty because U.S. beans are mixtures of a number of varieties, harvested with mechanical equipment. To overcome objections to use of mixed soybeans, we have studied improved methods for making miso from U.S. soybeans which presumably are applicable also to Japanese-grown beans. This approach involves removing the seedcoat and cracking the cotyledons into several pieces, but not into finely dispersed material or flour in which the fermentation does not occur.

Miso prepared from unsuitable beans has several objectionable features. The finished miso has unsightly spots caused by black or dark-colored hilums or colored seedcoats. In many varieties of soybeans undesirable flavors and odors are encountered and these appear to be derived from the seedcoats of the soybeans. Again, it is expensive to soak and cook whole soybeans for a protracted period. Even more undesirable is the fact that many soybeans do not take up water uniformly during the soaking and cooking process. Thus, beans are not evenly softened and the hard soybeans do not ferment properly. There is objectionable material in the form of insoluble fragments of hulls, which are not completely digested even over a period of 2 months. Miso, therefore, is not uniform in texture, appearance, or taste at the end of the fermentation. Another objection is the long fermentation time and the resultant expense of having many fermentation tanks tied up even though, as in the traditional process, the rice, inoculum, soybeans, and salt are thoroughly ground together to get reduced size, optimum dispersal, and thorough seeding of the substrate by the osmophilic microorganisms.

In the preparation of grits for our process soybeans are cleaned and then cracked by a roller-type crusher or other suitable apparatus. The hulls can then be readily removed or separated from the remainder of the soybeans. In our process grits represent approximately 2 to 10 pieces of a seed, minus its hull. If the grits are crushed into fine particles, they do not ferment as well as when they are left in bigger pieces. When coarse or fine grits regardless of the size are soaked in water, maximum water absorption occurs within 140 min. Actually soaking requires approximately only 1 hr, although we have used periods up to 5 hr with satisfactory results.

Soaked grits cook rapidly and evenly. When they are soaked for 1.5 hr and cooked with steam for 40 to 60 min at 7 psi, they are uniformly soft, rather elastic, and possess a highly desirable light color. When cooking lasts for 90 min, some darkening occurs. Besides preparing the beans for a rapid fermentation, the soaking and cooking effectively destroy microorganisms associated with soybeans.

When soybean grits are fermented, the fermentation proceeds at a greatly accelerated rate. In some cases, fermentation time has been reduced by 50%. Not only is the fermentation time reduced but the final product is more uniform in color and consistency. It is light yellow and also has a pleasant odor and flavor. Since the more fibrous portion of the seed is removed in soybean grits, the miso is higher in protein. At the same time, bad tastes and odors often associated with typical miso fermentations are eliminated. These several advantages bring about a superior food product at a reduced processing cost.

ACKNOWLEDGMENT

The senior author is indebted to the American Soybean Association for his expenses while in the United States and to the Foreign Agricultural Service for his travel expenses. The authors wish to thank the Japanese American Soybean Institute (Tokyo) for samples of Japanese soybeans and other Japanese products; Dr. A. K. Smith of the Oilseed Crops Laboratory for suggestions and assistance; Mr. A. M. Nash for preparing grits; Miss Margaret Shekleton for making the amino acid analysis; and Mr. Richard Gill (Pabst Brewing Company) for vitamin B_{12} assays.

SUMMARY

Small amounts of miso were fermented successfully in wide-mouthed glass jars of about 3,000 ml capacity. Miso was successfully prepared from U. S. soybeans by removing their seedcoats and fermenting the remainder of the beans which had been broken into several pieces (full-fat grits). When grits were used, the times required for soaking, cooking, and fermenting were all reduced. The miso prepared from fermented grits had excellent taste, color, and odor. A typical pilot-plant run of 17 kg is described.

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PREDICTING THE EFFECTIVENESS OF INDUSTRIAL ANTIMICROBIALS BY A PRACTICAL SCREENING PROGRAM

A. David Baskin and Jay C. Chapin

Scientific Chemicals, Inc.,* Chicago, Illinois

The quest for antimicrobial compounds must continue in order to satisfy the growing requirements of an industry and public educated to the practicability of mildewproofed and sanitized materials. Fungistatic and bacteriostatic compounds in current use are known to have one or more serious drawbacks with respect to color, leachability, instability to high ultraviolet intensities, incompatibilities with other additives, and toxicity to warm-blooded animals. Historically, these limiting factors aroused interest in a universal fungicide that would

(1) retain ideal physicochemical properties whatever the environmental stress;
(2) be biologically effective when applied to diverse materials;
(3) be of low cost; and
(4) be readily formulated in solvent, emulsion or dispersion systems.

For those of us intimately associated with prevention of deterioration, the universal fungicide is recognized now to be something of a fugitive myth The more realistic approach based on our experience is to define biocidal compounds in terms of the particular materials they will protect against microfloral attack. Thus, a given compound may afford excellent antimicrobial activities in cotton textiles yet be ineffective when incorporated in unsupported plastisols or paints, for example. Other compounds may afford good bacteriostasis and even be substantive to the materials to which they are applied but are so photosensitive that discoloration due to the interaction of compound and cloth in high energy light defeats their use in sanitizing finishes. If specific materials are to be protected selectively by particular compounds, the screening program is of fundamental importance in indexing chemical agents efficacious for the protection of certain materials. The choice afforded by the spectrum of activity so demonstrated permits the utmost flexibility for the selection of compounds with regard to their use. This flexibility is of considerable economic importance with respect to the market development of the biocide.

Various industrial organizations have amassed thousands of compounds synthesized in their laboratories over a period of years. Very little is known about these compounds apart from their basic chemical properties. Often, their structures and compositions suggest antimicrobial activity. The need is considerable to evaluate the potential effectivity of these compounds for industrial use in the most expeditious manner. Thus, the problem of screening is given, now, an air of immediacy and urgency. No longer may we be satisfied with some single test whose prime attribute is its rapidity. The information afforded by such a test may have little practical value because it is too restricted to demonstrate the usefulness of candidate antimicrobial compounds.

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*Formerly named: Scientific Oil Compounding Co., Inc.

Too often, collective efforts to establish screening procedures are defeated by the minutiae of personal preference. Well-intentioned committees must then adjourn to meet another day with the sad prospect of repeating their previous failures. Variations characteristic for a given material which is used as a substrate in biological testing often are mistaken for confounding results. Reproducibility of screening data is no less important than that of any other procedural problem; but reproducibility is possible, in any case, only in terms of the variation normal for the test material and/or biological agent being studied, as well as the constancy of the environmental stress being supplied. Fundamental statistical values such as standard deviation, error terms, and significance of data are important, therefore, to objectify the pattern of response evidenced.

As a working hypothesis, we propose the following definition for a screening procedure.

A screening procedure for the prediction of the effectiveness of industrial antimicrobials is a program composed of a judicious selection of relatively rapid tests affording measures of the broadest possible response to an arbitrarily varied biological spectrum and substrate. Upon the treated materials tested against a variety of microflora are imposed critical environmental stresses as the means for determining probable performance of the candidate biocidal compounds.

The data presented hereinafter are only by way of illustrating the kind of information developed in our screening program. No attempt will be made critically to evaluate the deficiencies or advantages of any candidate biocide.

The program we have achieved encompasses 4 phases:

1. THE DETERMINATION OF THE SOLUBILITY CHARACTERISTICS OF THE CANDIDATE COMPOUND

Routinely water and commonly available aliphatic and aromatic solvents are used (Table 1). Solution in more exotic solvents are studied also provided no more than partial solubility is obtained with the first group. Eventual development of a marketable biocide, however, is predicated on the cost of formulation, and expensive solvents would reduce its acceptability.

Table 1. Solution of Candidate Antimicrobial Compounds in Common Solvents by Weight of the Solvent Based on a 70% Wet Pick-up

Solvent	Specific gravity	g Cpd. per 25 ml solvent
water	1.0	0.375
mineral spirits	0.778	0.292
di-isobutylketone	0.809	0.303
isopropylalcohol	0.785	0.294

II. PRESUMPTIVE BACTERIOSTASIS AND FUNGISTASIS

Presumptive bacteriostasis is determined from streak plate cultures using *Micrococcus pyogenes* and *Escherichia coli.* For this test, desized cotton sheeting is treated with a volatile solution of the compound at a concentration of 1% by weight of the fabric using a 70% wet pick-up. Samples of this fabric are placed in the bacterial cultures, and zones of inhibition are measured after 20 to 24 hr of incubation at 35 C. Similar samples are placed on a dextrose — mineral-salts agar and inoculated with a suspension of *Aspergillus niger* spores. Zones of inhibition are measured after 3 days and again after 7 days of incubation. A presumptive measure of fungistasis is afforded by the persistence of this zone of inhibition. This is corroborated by the resistance to *Chaetomium globosum* of the treated material (as the sole source of carbon) placed on a mineral-salts agar and incubated for 14 days at 30 C. Strips of the same material are exposed to an ultraviolet lamp for 10 and 25 hr to determine the photosensitivity (indicated by a color change, Table 2) of the treated cloth to these wavelengths of light.

Table 2. Discoloration* of Treated Materials to Carbon Arc Source Ultraviolet with No Water Cycling

3.0 oz Cotton sheeting treated with:	Color change† following hr exposed	
	10 hr	25 hr
7284	0	1
2174	0	1
A-151	1	2
2722	3	3
B-622	0	1
4030	0	1
5600	0	1
6618	2	3
tributyltinoxide	3	3
phenyl Hg propionate	3	4
untreated	0	0

*Darkening or bleaching of the exposed fabric compared to the unexposed portion of the same sample.
†Color change index: 0, no change from that of the treated material; 1, trace change; 2, slight change; 3, moderate change; 4, intensive change.

III. SUBSTANTIVITY, BACTERIOSTASIS, AND FUNGISTASIS

If the fabric treated with the candidate biocide does not discolor in the ultraviolet light and is bacteriostatic, the substantive nature of the compound is measured.

We recognize a compound to be substantive if after reaction with cotton cellulose the "physical bonding" of the chemical to the cloth is such as to resist removal after successive laundering cycles. In our laboratory 20 laundering cycles serves as the means, and insignificant change in biological effectivity following laundering as the criterion, for substantivity.

The substantive reaction is accomplished as a batch operation in which, for example, 3-oz desized cotton sheeting is placed in a solution of the compound, the temperature of which is at the boil maintained for 10 min. Four other samples of the same fabric are treated successively in the same bath repeating the reaction temperature — time factor for each sample. In this manner an empirical measure is obtained of the degree of exhaustion achieved from the bath. All treated fabrics are then laundered for 20 cycles, each of which is a 5 min wash at 140 F using a neutral soap. Subsamples are taken after 5, 10, 15, and 20 washings. Bacteriostasis is then measured for each of these subsamples as well as the unlaundered material. If the fabric first treated in this manner remains bacteriostatic after all launderings and the others do not inhibit the Gram-negative and/or Gram-positive test bacteria it would be concluded, in this instance, that the compound was substantive for cellulose and that exhaustion occurred during the first treatment.

Fabric samples treated with the candidate compound are then exposed for 25 and 100 hr to the high ultraviolet intensities of a carbon arc and the full water cycle in an Atlas Weatherometer (Table 3). This is followed by the bacterial culture test described previously.

Table 3. Nearest Whole % Change in Breaking Strength Due to Weathering* and/or Soil Burial† of 8-oz Cotton Duck Treated with Candidate Biocides at 1% by Weight of the Fabric

Sample treated with:	As treated	Weathered 100 hr	Soil burial 4 weeks	Weathered 100 hr, soil burial 4 wks.	General evaluation
7284	+ 3	−30	−100	− 52	fails
2174	+10	− 9	− 8	− 8	passes
A-151	0	−15	− 17	− 42	borderline
2722	+ 2	−27	− 3	−100	fails
B-622	+ 3	−34	− 53	− 82	fails
4030	0	−23	−100	− 64	fails
5600	+ 2	−19	− 56	−100	fails
tributyltin-oxide	+ 2	−21	− 8	−100	fails
phenyl Hg propionate	+ 2	−19	−100	−100	fails
untreated	− ‡	−17	−100	−100	fails

*Atlas Weatherometer, carbon arc source ultraviolet, and full water cycle.
†Soil burial per Method 5762, CCC-T-191.
‡All values computed from the breaking strength of the untreated material prior to weathering and/or soil burial.

If the candidate compound proves to be only fungistatic it is padded on to 8-oz cotton duck, leached per Method 5830, and buried in soil for 14 days per Method 5762, of Federal Specification CCC-T-191 (1951). The measure of change in breaking strength is considered diagnostic of the potential efficacy of the compound as a fabric preservative.

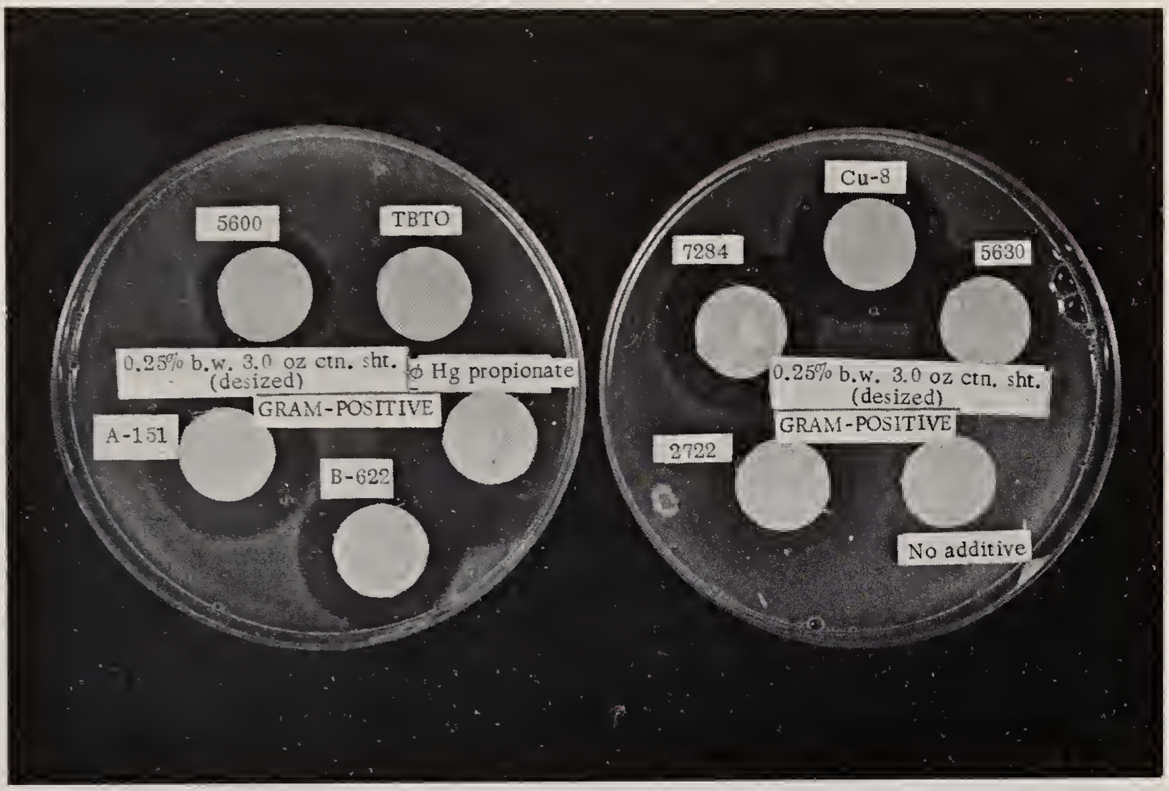
5600
TBTO
0.25% b.w. 3.0 oz ctn. sht. (desized)
ϕ Hg propionate
GRAM-POSITIVE
A-151
B-622
Cu-8
7284
5630
0.25% b.w. 3.0 oz ctn. sht. (desized)
GRAM-POSITIVE
2722
No additive

Fig. 1. Bacteriostasis of candidate biocide compounds applied to 3.0-ounce cotton sheeting tested against *Micrococcus pyogenes.* AATCC Tentative Test Method 90–1958 (1959).

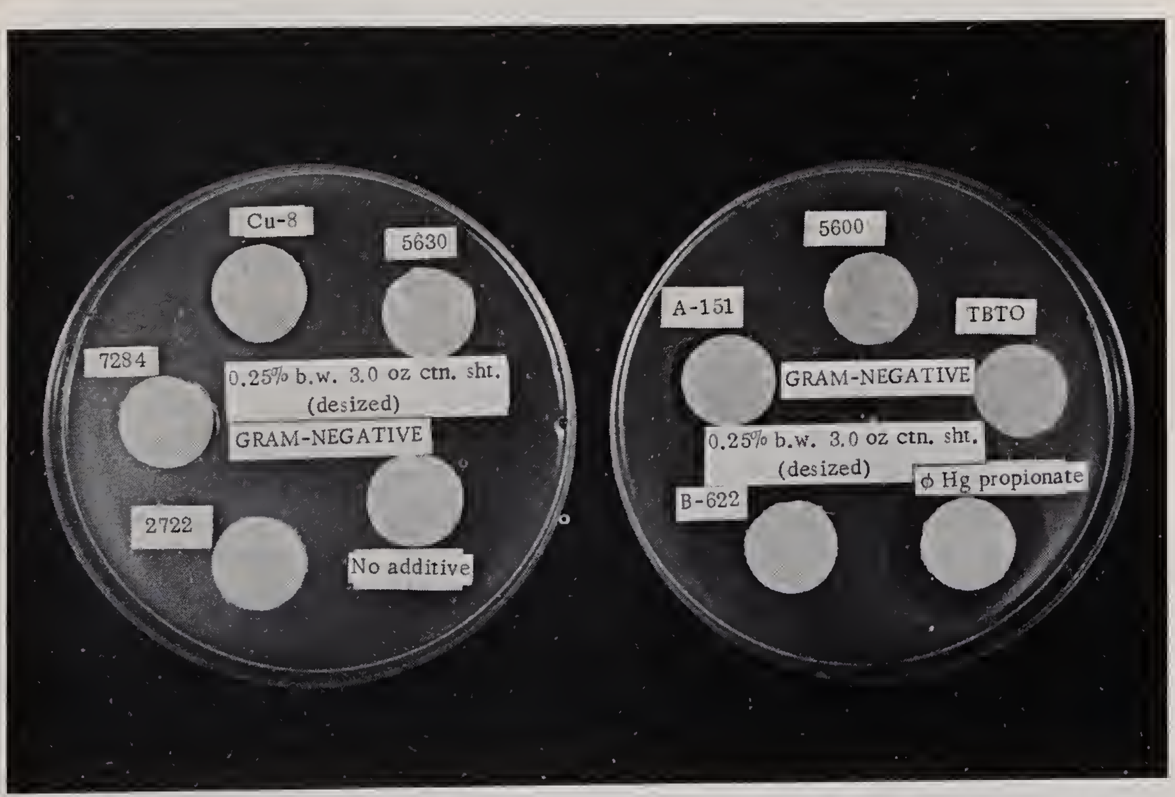
Cu-8
5630
7284
0.25% b.w. 3.0 oz ctn. sht. (desized)
GRAM-NEGATIVE
2722
No additive
5600
A-151
TBTO
GRAM-NEGATIVE
0.25% b.w. 3.0 oz ctn. sht. (desized)
ϕ Hg propionate
B-622

Fig. 2. Bacteriostasis of candidate biocide compounds applied to 3.0-ounce cotton sheeting tested against *Escherichia coli.* AATCC Tentative Test Method 90–1958 (1959).

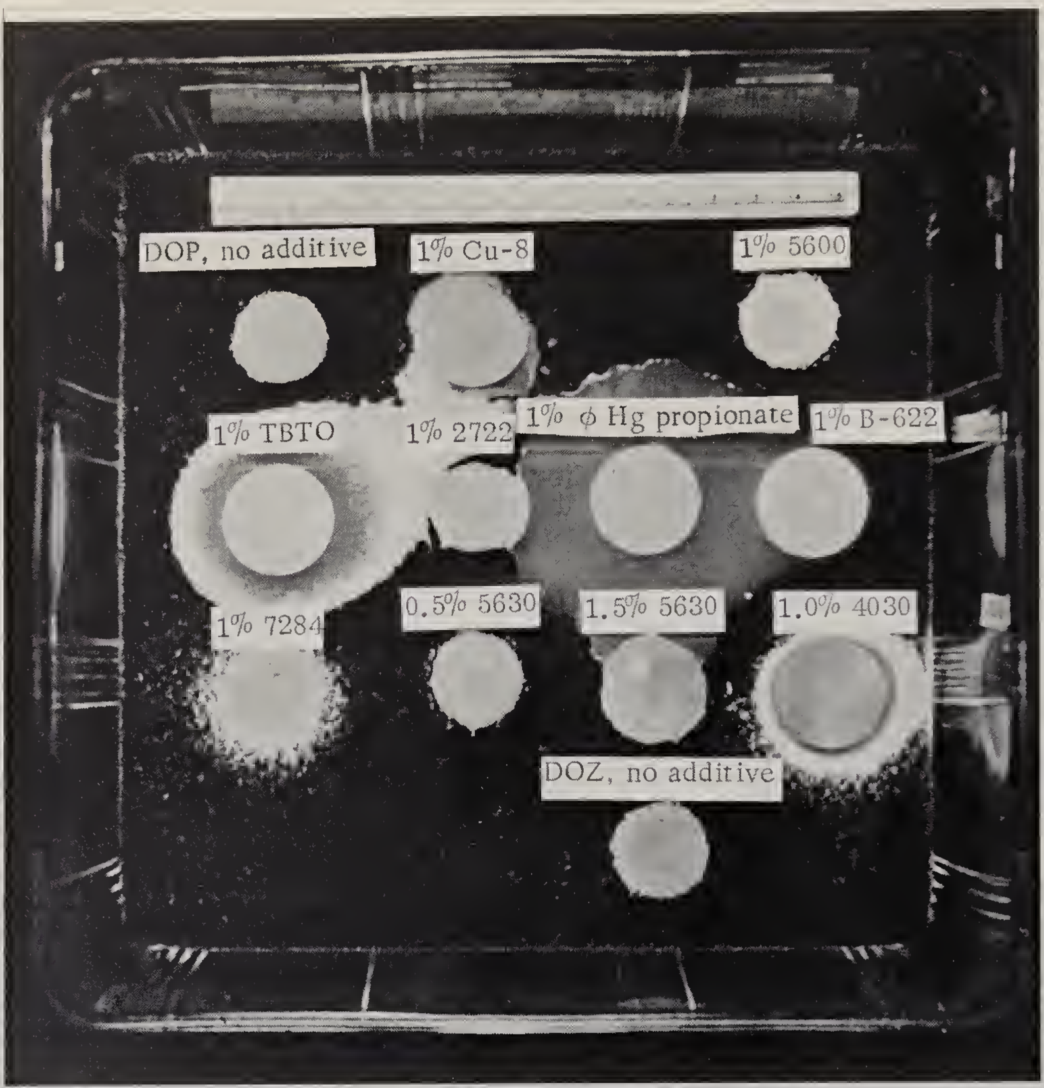

Fig. 3. Plastisol treated with candidate biocide compounds (1% by weight of the plastisol formulations) tested against *Aspergillus niger*.

IV. APPLICATIONS

Compounds that have survived all the tests for bacteriostasis are applied to cotton scrim, sheeting, cotton duck, cotton linters, flannelette and wool. Laundering when required is followed by bacteriostatic tests in accord with the AATCC Tentative Test Method 90-1958 (1959) (Figs. 1 and 2), a pour plate procedure, and the titration method devised by Majors (1959). Bacteriostatic compounds are incorporated in plastisols drawn to 8 mil and 30 mil films and cured at 160 C. Slush molds are also prepared and the bacteriostatic properties of these plastics are measured as before. For this purpose melting point and decomposition temperatures of the compound are determined, and a polyvinyl chloride resin is plasticized with DOZ or DOP and stabilized with a barium-cadmium system in such proportions as to achieve the best possible heat stability for the treated plastic. Heat stability is measured by an accelerated aging test at 195–200 C.

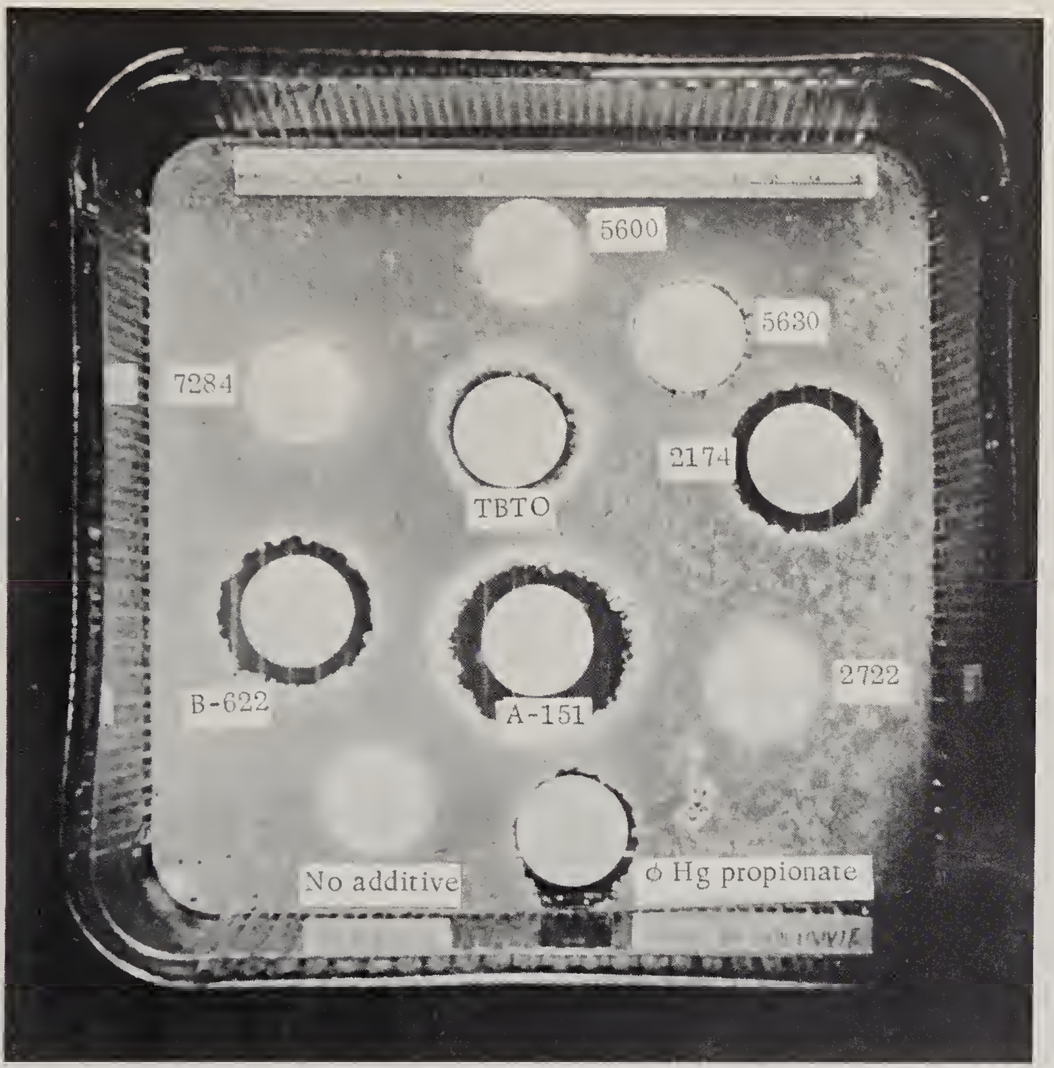

Fig. 4. Zinc-free oil-base paint films on Whatman No. 4 filter paper containing candidate biocide compounds (1% by weight of the paint) tested against *Penicillum citrinum.*

Compounds that have survived all fungistatic tests are used to treat several weights of cotton duck, plastisols (Fig. 3), vinyl-coated cotton duck, zinc-free oil-based paint (Figs. 4 and 5) by the Method of Vicklund and Manowitz (1949), and wood (as a dip treatment). Appropriate physical tests such as Clark flexibility, tear strength, bending moments of force, breaking strength and visual indices of growth are made to evaluate the susceptibility of these materials. Candidate compounds surviving these tests are formulated as emulsions, dispersions, or solutions in both aqueous and solvent systems. Performance trials are then arranged with leading manufacturers and processors to determine whether the potential of the compound as demonstrated in the laboratory is fulfilled by commercial processing. The value of these cooperative tests with industry cannot be underestimated.

The procedures reviewed have been arranged as a factorial design experiment. This makes possible an economy of material to be tested in the shortest possible time, a predetermined flow sheet of the data required, the elimination

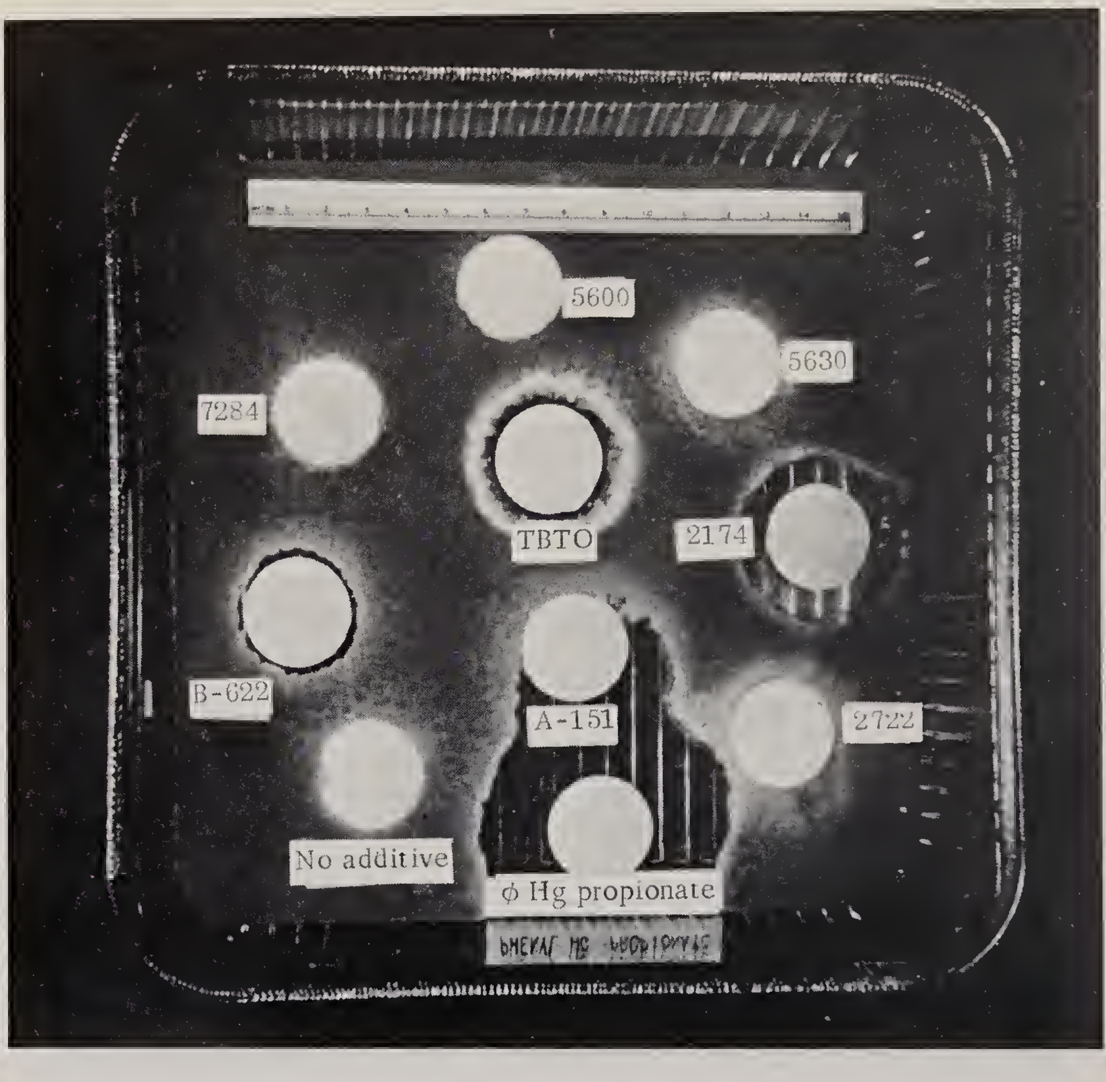

Fig. 5. Zinc-free oil-base paint films on Whatman No. 4 filter paper containing candidate biocide compounds (1% by weight of the paint) unweathered, tested against *Aspergillus oryzae.*

of candidate compounds at any time because of serious deficiencies, and the maximum amount of information with regard to the biological effectivity of a compound. All the procedures used are not short term tests but they comprise the most rapid and diagnostic methods we find applicable to a comprehensive screening program for industrial antimicrobials.

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PHYLOGENY OF ACTINOMYCETES AS REVEALED BY SUSCEPTIBILITY TO ACTINOPHAGE *

S. G. Bradley, D. L. Anderson, and L. A. Jones

Department of Bacteriology, University of Minnesota, Minneapolis, Minnesota

All living beings cannot be arranged unequivocally in an orderly and logical taxonomic system on the basis of comparative morphology alone. For this reason, physiological capabilities, chemical composition, genetic interactions, and comparative parasitology have become increasingly important in systematics. Orders and sometimes suborders of birds, for example, have their own peculiar tapeworm fauna; these highly specialized, host-specific parasites are of value in avian classification. Studies of the host ranges of parasites such as pinworms, sclerostomes, and malarial parasites (*Plasmodium* spp.) may be of taxonomic value if correlated with more extensive and accurate morphological studies (Cameron, 1952). Similarly, parasitic fungi can be used as aids for classifying flowering plants (Savile, 1954). Host specificity, therefore, can serve as one basis for speculation about phylogeny and systematics of multicellular organisms.

Susceptibility to bacteriophage has proven a useful adjunct in delineation of genera and species of bacteria and in identifying closely related strains of a single species. Host range has been applied as a criterion for classification of strains of *Corynebacterium diphtheriae* (Keogh *et al.*, 1938), of *Staphylococcus aureus* (Fisk, 1942; Fisk and Mordvin, 1944) and of soil bacteria (Conn *et al.*, 1945). Furthermore, phage typing has been employed with many other groups of bacteria, including streptococci and enteric bacteria. The value of phage typing is increasing as additional phages and improved techniques become available. Moreover, studies of these parasites may provide significant information about the origins and interrelationships of microbes as well as facilitate their identification.

Wieringa and Wiebols (1936) were the first investigators to report that actinomycetes were lysed by transmissible, filtrable agents similar to bacteriophages. Subsequently, transmissible agents which attacked antibiotic-producing cultures of *Streptomyces griseus* were isolated from antibiotic beers (Saudek and Colingsworth, 1947; Woodruff, Nunheimer, and Lee, 1947). The lytic agents were particulate and resembled *Escherichia coli* bacteriophage morphologically. The specificity of some actinophages focused attention on their potential use for identification of actinomycetes, for example, Welsch *et al.* (1957) isolated species-specific streptomycete-phages. In order to obtain additional suitable indicator viruses, methods have been sought for deriving streptomycete-phages of limited

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Table 1. Hosts

Strains were grown at 28C for 11 days on a complex medium composed of 10 g glucose, 5 g Difco peptone, 3 g Difco yeast extract, 1 g Difco casitone, 0.5 g Difco bacto beef, 15 g Difco agar and 1 L deionized water. The medium was adjusted to pH 7 with NaOH prior to autoclaving. Colors are according to Ridgeway (1912).

Strain	Origin	Color of Mycelium		Color of Soluble Pigment	Colony Texture	Arrangement of Spores
		Upperside	Underside			
S. griseus S104	E. McCoy, Univ. Wisconsin	pale olive-buff	cinnamon-rufous	Mars yellow	tenacious	straight
S. cyaneus S45	Mutant of S32; S32 from Ill. Soil Sample	greenish-glaucous-blue	olivaceous black	Saccardo's olive	tenacious	spiraled
S. violaceoruber S199	Nature, Wisc. Soil Sample	pale gull gray	bluish black	Berlin blue	tenacious	spiraled
S. violaceoruber S16	NRRL-B-1257 U.S. Dept. Agric.	pale gull gray	bluish black	Delft blue	tenacious	spiraled
S. violaceoruber Sermonti 1	G. Sermonti, Rome, Italy	pale gull gray	bluish black	Delft blue	tenacious	spiraled
S. griseus 1945	E. McCoy, Univ. Wisconsin	olive-buff	orange-citrine	none	tenacious	straight
S. olivaceus S11	J. Lederberg	pale olive-buff	honey yellow	none	tenacious	straight
S. vinaceus S208	Nature, Minn. Soil Sample	pale smoke gray	dahlia carmine	dark dull purple	tenacious	straight
N. paraguayensis N303	N. F. Conant, Duke Univ.	wood brown	wood brown	none	tenacious	straight
Micromonospora sp. A401	Univ. Minnesota Culture Collection	Sudan brown	Sudan brown	none	tenacious	singly
Nocardia sp. 3403	E. L. Schmidt, Univ. Minnesota	ochraceous buff	ochraceous buff	none	soft	none
N. brasiliensis N301	N. F. Conant, Duke Univ.	tilleul-buff	chamois	none	tenacious	straight

S. violaceoruber S133	Mutant of Sermonti 5; Sermonti 5 from Sermonti 1	lavender gray	bluish black	bluish black	tenacious	spiraled
N. madurae N302	N. F. Conant, Duke Univ.	marguerite yellow	marguerite yellow	none	tenacious	none
N. corallina W5	J. B. Clark, Univ. Oklahoma	cartridge buff	cream-buff	none	soft	none
Actinoplanes sp. P15	J. N. Couch, Univ. N. Carolina	cream-buff	Naples yellow	none	tenacious	motile spores in sporangia
Streptosporangium sp. 27b	J. N. Couch, Univ. N. Carolina	purplish gray	claret brown	brick red	tenacious	nonmotile spores in sporangia
M. phlei A400	Univ. Minnesota Culture Collection	straw yellow	straw yellow	none	soft	none
N. asteroides N300	N. F. Conant, Duke Univ.	maize yellow	maize yellow	none	tenacious	straight
S. griseus S34	Nature, Wisc. Soil Sample	pale olive-buff	olive-brown	none	tenacious	straight
S. griseus S86	Nature, Wisc. Soil Sample	tilleul-buff	ochraceous tawny	ochraceous tawny	tenacious	straight
S. griseus 3475	S. A. Waksman, Rutgers Univ.	pale olive-buff	snuff brown	Mars yellow	tenacious	straight
S. griseus 3511	S. A. Waksman, Rutgers Univ.	pale olive-buff	orange-citrine	none	tenacious	straight
S. griseus 1947	E. McCoy, Univ. Wisconsin	pale olive-buff	Mars yellow	Mars yellow	tenacious	straight
S. venezuelae S13	J. Lederberg	pale olive-buff	argus brown	Mars yellow	tenacious	straight

host range from polyvalent viruses (Shirling, 1959). Specificity of each of four phages for a single species was claimed as a result of several successive subcultures on particular hosts. St. Clair and McCoy (1959), using genus-specific streptomycete-phages, reported that plaque morphology could be used to identify both host and phage. Polyvalent actinophages, some of which attacked both nocardiae and streptomycetes, have been used as an index for biological affinity (Bradley and Anderson, 1958). Inasmuch as most of the streptomycetes were not readily distinguishable from the nocardiae on morphological grounds either, the correctness of placing these strains into different genera and families has been challenged.

In the present study, 4 temperate and 33 virulent actinophages have been isolated; the host range of each, with respect to 25 actinomycetes has been determined. Tentative phylogenetic relationships have been proposed on this basis.

MATERIALS AND METHODS

Strains and Media

Species selected from six genera of Actinomycetales were employed in this investigation. Although most of these microbes were members of the genera *Nocardia* and *Streptomyces*, strains of *Micromonospora, Mycobacterium, Actinoplanes* and *Streptosporangium* were studied also. The source and morphological characteristics of microbes used are summarized in Table 1. Stock cultures were maintained on complete medium agar of the following composition, solution I: 20 g glucose, 1 g Difco yeast extract, 1 g Difco casitone, 15 g Difco agar, 0.5 g $MgSO_4 \cdot 7H_2O$, and 500 ml deionized water; solution II: 2 g KNO_3, 2 g Na_2HPO_4, and 500 ml deionized water. Solutions I and II were autoclaved separately and mixed before the milieu was dispensed. Stocks were subcultured by mass inoculation of fresh medium with aerial and/or vegetative hyphae. Isolation of actinophage and tests for susceptibility were carried out with peptone — yeast extract medium composed of 5 g Difco peptone, 3 g Difco yeast extract, 0.5 g $Ca(NO_3)_2$, and 1 L deionized water. Solid milieux were prepared by adding 1.5% Difco agar. All media were autoclaved at 121 C for 15 min.

Isolation of Actinophages

Actinophages were isolated from soil samples of diverse origin by the following procedure: An Erlenmeyer flask (125 ml) containing 20 to 30 ml of sterile peptone — yeast extract broth was inoculated with prospective host organism (10^8–10^9 plating units per ml) and with approximately one teaspoonful of a soil sample. The mixture was incubated, with shaking, for 24 hr at 30 C. Following incubation, the contents of the flask were centrifuged to remove the soil and host material; then the supernate was filtered through an unglazed porcelain filter to render it bacteriologically sterile. The filtrate was transferred to sterile vials and chloroform was added to prevent subsequent bacterial contamination. Finally, the filtrate was tested for the presence of bacterial virus by spreading 0.05 ml of filtrate with 10^7–10^8 plating units of the host organism on peptone — yeast extract medium agar. Because confluent growth was required for accurate enumeration of actinophage plaques, tough, tenacious, asporogenous mycelia were crushed in a Potter-Elvehjem tissue homogenizer thereby producing a uniform inoculative suspension. The development of plaques after incubation at 30 C for 1 to 5 days was considered evidence for the existence of phage particles in the particular filtrate. Phages isolated in this manner were purified by 4 to 5 serial passages from single plaques. Stock lysates were prepared in broth, clarified by centrifugation, freed of bacteria by filtration, and stored at 4 C (Table 2).

Table 2. Isolation and Distribution of Actinophages

Origin of Soil Sample	Presumptive Hosts Tested	Actinophage Isolated
Blue Heron Plaza, Ohio	*S. vinaceus* S208 *N. corallina* W5 *Micromonospora* sp. A401 *Streptosporangium* sp. 27b *S. violaceoruber* S199	MSP-20 MSP-19
New Stanton, Pennsylvania	*S. vinaceus* S208 *Micromonospora* sp. A401	MSP-21 MMP-1
Ohio Turnpike	*S. vinaceus* S208 *Micromonospora* sp. A401	MSP-22
Wisconsin Sandy Garden	*Micromonospora* sp. A401 *S. violaceoruber* S199 *N. corallina* W5	MSP-18 MNP-8
Mississippi River Mud, Minnesota	*Actinoplanes* sp. P15 *Streptosporangium* sp. 27b *S. violaceoruber* S199 *N. corallina* W5	MMP-2
Caoba Silty Loam, Central America	*S. vinaceus* S208 *Actinoplanes* sp. P15 *Streptosporangium* sp. 27b *S. violaceoruber* S199 *N. corallina* W5	MMP-3
Palos Blancos Clay, Central America	*N. brasiliensis* N301 *S. vinaceus* S208 *S. violaceoruber* S199 *N. corallina* W5	MNP-6
Minnesota Lawn Soil	*Micromonospora* sp. A401 *N. brasiliensis* N301 *S. violaceoruber* S199 *N. corallina* W5	MNP-7 MSP-15
Minnesota Garden Soil	*Micromonospora* sp. A401 *S. violaceoruber* S199 *N. corallina* W5	MSP-16
Minnesota Lawn Soil	*Micromonospora* sp. A401 *S. violaceoruber* S199 *N. corallina* W5	MSP-17
Univ. Minnesota Campus	*S. violaceoruber* S199 *S. griseus* S104 *N. paraguayensis* N303 *N. brasiliensis* N301 *N. madurae* N302 *Nocardia* sp. 3403	MNP-4

Table 2 (continued)

Origin of Soil Sample	Presumptive Hosts Tested	Actinophage Isolated
Minnesota Garden Soil	*S. violaceoruber* S199	
	S. griseus S104	MSP-8
	N. paraguayensis N303	
	N. brasiliensis N301	MNP-1
	N. madurae N302	
	Nocardia sp. 3403	
Mississippi River Mud, Minnesota	*S. violaceoruber* S199	MSP-7
	S. griseus S104	MSP-9
	N. paraguayensis N303	
	N. brasiliensis N301	MNP-2
	N. madurae N302	
	Nocardia sp. 3403	MNP-5
Minnesota Barnyard	*S. violaceoruber* S199	
	S. griseus S104	
	N. paraguayensis N303	MNP-3
	N. brasiliensis N301	
	N. madurae N302	
	Nocardia sp. 3403	
Minnesota Compost	*S. violaceoruber* S199	MSP-10
	N. asteroides N300	
	Streptosporangium sp. 27b	
	Actinoplanes sp. P15	
	Micromonospora sp. A401	
	N. brasiliensis N301	
Sherborn, Mass. Garden Soil	*S. violaceoruber* S199	MSP-11
	N. asteroides N300	
	Streptosporangium sp. 27b	
	Actinoplanes sp. P15	
	Micromonospora sp. A401	
	N. brasiliensis N301	
Univ. Wisconsin Campus A	*S. griseus* S104	WSP-1
Univ. Wisconsin Campus B	*S. griseus* S104	WSP-2
Univ. Wisconsin Campus C	*S. griseus* S104	WSP-3
Univ. Wisconsin Campus D	*S. griseus* S104	WSP-4
Univ. Wisconsin Campus E	*S. griseus* S104	WSP-5
Univ. Wisconsin Campus F	*S. griseus* S104	V_{104}
Mississippi River Mud, Minnesota A	*S. griseus* S104	MSP-1
Mississippi River Mud, Minnesota B	*S. griseus* S86	MSP-2
Mississippi River Mud, Minnesota C	*S. griseus* S104	MSP-3
Mississippi River Mud, Minnesota D	*S. griseus* S104	MSP-4
Mississippi River Mud, Minnesota E	*S. griseus* S104	MSP-5

The temperate phages (MLSP series) were derived from the following lysogenic stocks: MLSP-1 from *S. griseus* 1947; MLSP-2 from *S. griseus* 1945; MLSP-3 from *S. griseus* S104; MLSP-4 from *S. olivaceus* S11.

Susceptibility Test System

Susceptibility to actinophage was determined on peptone – yeast extract agar by spreading a measured aliquot of a suspension containing 10^7–10^9 viral particles per ml, as determined on the original host, with an inoculum of prospective host organism sufficient to yield confluent growth. Control plates contained only the host without added phage particles. The appearance of plaques on test plates after incubation at 30 C for 1 to 5 days indicated susceptibility of the test organism to the phage. Sensitivity was confirmed by serial subculture of actinophage taken from single plaques.

Table 3. Antibiotic Susceptibilities of Actinomycetes

Strains	Antibiotics								
	oxytetracycline	*novobiocin*	*carbomycin*	*chlortetracycline*	*bacitracin*	*streptomycin*	*neomycin*	*penicillin*	*nystatin*
S. griseus S104	20	5	5	25	>5	>50	5	500	>100
S. violaceoruber S199	200	50	5	25	5	5	0.5	500	>100
S. vinaceus S208	200	5	5	25	>5	0.5	5	50	>100
M. phlei A400	20	50	5	25	5	50	5	500	>100
N. corallina W5	20	500	5	25	0.5	0.5	5	0.5	>100
N. brasiliensis N301	20	500	5	25	>5	>50	500	500	>100
N. asteroides N300	20	5	5	25	5	>50	5	500	>100
Actinoplanes sp. P15	20	500	5	250	>5	50	50	500	>100
Streptosporangium sp. 27b	20	5	5	25	>5	>50	50	>500	>100
Micromonospora sp. A401	20	500	5	250	0.05	50	5	0.5	>100

Concentrations of all antibiotics are expressed as μg/ml complete medium agar, with the exception of bacitracin and penicillin (units/ml complete medium agar).

Susceptibility of Actinomycetes to Antibiotics

The susceptibilities of ten actinomycetes to antibacterial and antifungal antibiotics were determined by streaking the strains on complete medium agar supplemented with varying drug concentrations. Antibiotics used included oxytetracycline (Terramycin, Pfizer), novobiocin (Albamycin, Upjohn), carbomycin (Magnamycin, Pfizer), chlortetracycline (Aureomycin, Lederle), bacitracin (Upjohn), dihydrostreptomycin (Cal. Corp. Biochem. Res.), neomycin (Cal. Corp. Biochem. Res.), penicillin G (Lederle) and nystatin (Mycostatin, Squibb).

Table 4. Actinophage Host Ranges

Host	Phage														
	WSP 1	WSP 2	WSP 3	WSP 4	WSP 5	V_{104}	MLSP 1	MLSP 2	MLSP 3	MLSP 4	MSP 1	MSP 2	MSP 3	MSP 4	MSP 5
S. griseus S34	-	+	-	-	-	-	-	-	-	+	-	-	+	+	+
S. griseus S86	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
S. olivaceus S11	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-
S. griseus 3475	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
S. griseus 3511	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+
S. cyaneus S45	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
S. griseus 1947	+	+	+	+	+	+	+	+	+	*	+	-	+	+	+
S. griseus 1945	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
S. griseus S104	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
S. violaceoruber S16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. violaceoruber S133	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-
S. venezuelae S13	+	-	+	+	+	*	+	+	-	*	+	+	+	+	*

+ Susceptibility of the actinomycete to actinophage as revealed by plaque formation.
- Resistance of the actinomycete to actinophage.
* Not tested.

Table 5. Actinophage Host Ranges

Host	Phage																					
	MSP 7	MSP 8	MSP 9	MSP 15	MSP 16	MSP 17	MSP 18	MSP 19	MSP 20	MSP 21	MSP 22	MMP 1	MMP 2	MMP 3	MNP 1	MNP 2	MNP 3	MNP 4	MNP 5	MNP 6	MNP 7	MNP 8
S. griseus S104	-	+	+	+	+	+	+	+	-	+	-	+	-	-	-	-	+	+	+	+	-	-
S. cyaneus S45	-	-	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-
S. violaceoruber S199	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. violaceoruber S16	*	*	*	+	-	+	+	+	-	+	-	-	-	-	*	*	*	*	*	-	-	-
S. violaceoruber Sermonti 1	*	*	*	+	+	+	+	+	-	+	-	-	-	-	*	*	*	*	*	+	-	-
S. griseus 1945	-	+	-	+	+	+	+	+	-	+	+	-	-	-	-	-	+	+	-	+	-	-
S. olivaceus S11	-	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	+	-	+	+	-
S. vinaceus S208	*	*	*	-	-	-	-	-	+	+	+	-	-	-	*	*	*	*	*	-	-	-
N. paraguayensis N303	-	+	-	+	+	+	+	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-
Micromonospora sp. A401	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Nocardia sp. 3403	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
N. brasiliensis N301	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-
N. madurae N302	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
N. corallina W5	*	*	*	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	-	-	+
Actinoplanes sp. P15	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
Streptosporangium sp. 27b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M. phlei A400	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N. asteroides N300	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ Susceptibility of the actinomycete to actinophage as revealed by plaque formation.
- Resistance of the actinomycete to actinophage.
* Not tested.

RESULTS

The Actinomycetales are considered closely related to Eubacteriales inasmuch as the cells of both are only 0.5 to 3 μ in diameter (Waksman, 1950), because the cell walls of actinomycetes and true bacteria have similar chemical compositions (Cummins and Harris, 1956a, 1956b), and because strains from both groups are vulnerable to lysozyme (Sohler *et al.*, 1958; Bradley, 1959a, 1959b). Additionally, members of these two orders are susceptible to antibacterial antibiotics, for example, all actinomycetes studied here were markedly inhibited by carbomycin (Table 3). However, there were substantial differences in the magnitude of drug sensitivity of particular species; with respect to penicillin, *Nocardia corallina* was inhibited by 0.5 unit/ml whereas 500 units/ml were required to suppress growth of *Streptomyces griseus*. It should be noted though that there was as much variation in drug susceptibility within a given genus as there was among genera; *N. asteroides* was inhibited by 1/100 the concentration of novobiocin that was necessary to prevent growth of *N. brasiliensis*. The antifungal antibiotic nystatin did not inhibit any tested members of the genera *Streptomyces, Nocardia, Actinoplanes, Streptosporangium* or *Micromonospora*.

Using 25 actinomycetes, the host ranges of 37 actinophages were determined by the methods described previously (Tables 4 and 5). Four of 7 cultures labeled *S. griseus* displayed similar sensitivity to bacterial viruses. Interestingly, these *S. griseus* strains resembled *S. venezuelae* strain S13 more than strain 1945 which was reported to be a member of the former species. Recent studies, however, demonstrated that some cultures designated *S. griseus*, probably including strain 1945, were in reality strains of *S. coelicolor* (Kutzner and Waksman, 1959). On the basis of susceptibility to actinophage, *S. griseus, S. cyaneus, S. violaceoruber, S. olivaceus,* and *N. paraguayensis* were related. It should be noted that a nocardia was included in this grouping. In this instance, there seemed to be little doubt but that the true biological affinities of *N. paraguayensis* N303 were with the streptomycetes rather than with "true" nocardiae such as *N. asteroides* and *N. corallina*. Significantly, *Nocardia* sp. 3403 and *S. griseus* S104 were attacked by actinophage MNP-5, *N. brasiliensis* N301 and *S. griseus* S104 were sensitive to phage MNP-6, and phage MNP-7 was virulent for *S. olivaceus* S11 and *N. brasiliensis* N301. *N. brasiliensis* strain N301 was weakly acid-fast and *Nocardia* sp. 3403 was typical of the waxy, fragmenting "true" nocardiae, therefore these intergeneric cross-susceptibilities could not be attributed merely to incorrectly labeled cultures. Because relationships among some actinomycetes were not apparent immediately from the data presented, the strains were grouped on the basis of common sensitivity or resistance to individual viruses. Both *S. griseus* strain S34 and *S. griseus* strain 3511 were susceptible to 4 of 15 actinophages, immune to 10 actinophages and differed with respect to only 1 (Table 4). *S. cyaneus* S45 and *S. griseus* 3511 behaved the same with respect to 12 of 15 actinophages, and strains S45 and S34 acted alike in 11 of 15 tests; therefore, *S. griseus* strains S34 and 3511 were considered more closely allied than S34 and *S. cyaneus* strain S45, or 3511 and S45. Strains 3511 and S45 seemed to be slightly more alike than S45 and S34. Extending this analysis, phylogenetic relationships for other actinomycetes studied here have been proposed (Fig. 1). It is interesting to note that strains designated *S. griseus* formed a continuous series on the basis of susceptibility to actinophage.

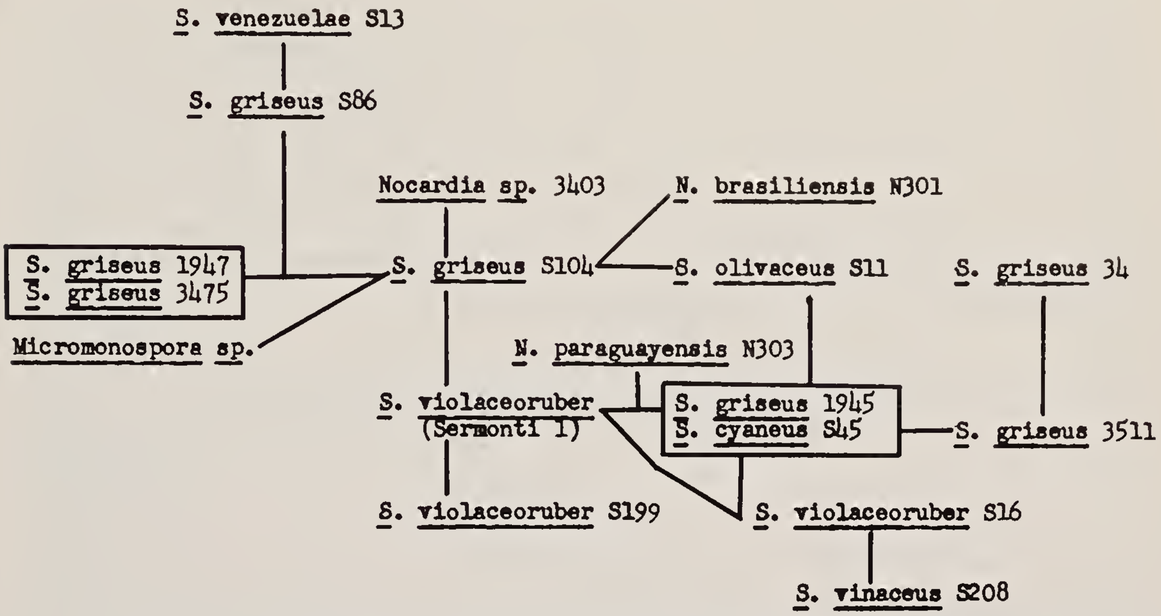
S. venezuelae S13
S. griseus S86
Nocardia sp. 3403
N. brasiliensis N301
S. griseus 1947
S. griseus 3475
S. griseus S104
S. olivaceus S11
S. griseus 34
Micromonospora sp.
N. paraguayensis N303
S. violaceoruber (Sermonti 1)
S. griseus 1945
S. cyaneus S45
S. griseus 3511
S. violaceoruber S199
S. violaceoruber S16
S. vinaceus S208

Fig. 1. Relationship among actinomycete hosts on the basis of virus susceptibility. Strains enclosed in boxes cannot be arranged sequentially on the basis of susceptibility to phages tested.

Similarly, host range was indicative of phylogenetic relationships among the actinophages (Fig. 2). Phages attacking *S. vinaceus* (MSP-20, MSP-21 and MSP-22)

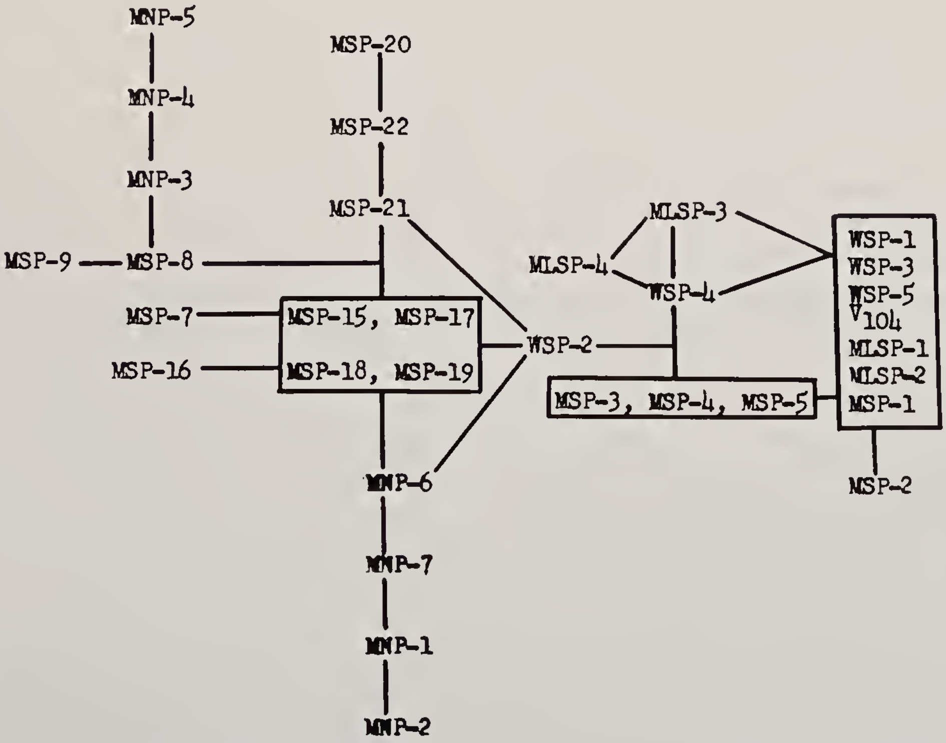
MNP-5
MSP-20
MNP-4
MSP-22
MNP-3
MSP-21
MLSP-3
MSP-9
MSP-8
MLSP-4
WSP-4
WSP-1
WSP-3
WSP-5
V104
MLSP-1
MLSP-2
MSP-1
MSP-7
MSP-15, MSP-17
WSP-2
MSP-16
MSP-18, MSP-19
MSP-3, MSP-4, MSP-5
MMP-6
MSP-2
MMP-7
MMP-1
MMP-2

Fig. 2. Relationship among actinophages as revealed by cross susceptibilities of actinomycete hosts. Phages labeled MSP and WSP denote *Streptomyces* phages isolated, the MNP series indicates *Nocardia* phages, while MMP designates phages virulent for genera of Actinomycetales other than *Streptomyces* and *Nocardia*. The group of phages named MLSP includes temperate viruses of *Streptomyces*.

and phages isolated against *N. brasiliensis* N301 (MNP-6, MNP-7, MNP-1 and MNP-2) were related closely to phages MSP-15, 16, 17, 18 and 19, which were virulent for *S. violaceoruber*. Additionally, *Nocardia* phages MNP-3, MNP-4, and MNP-5 had hosts in common with *Streptomyces griseus* phage MSP-8. Moreover, streptomycete-phage WSP-2 was effective against many of the same actinomycete hosts as *N. brasiliensis* phage MNP-6, *S. violaceoruber* phages MSP-15, 16, 17, 18, and 19 and *S. vinaceus* phages MSP-20, 21, and 22. These findings indicated that viruses isolated against strains of *Streptomyces* and *Nocardia* often had similar host ranges and therefore were related.

Table 6. Efficiency of Isolation of Actinophages

Host	Number Soil Samples* Tested	Number Soil Samples Containing Actinophage
S. violaceoruber S199	41	8
S. griseus S104	16	12
N. paraguayensis N303	6	1
N. brasiliensis N301	35	4
N. madurae N302	6	1
Nocardia sp. 3403	6	1
N. asteroides N300	24	0
Streptosporangium sp. 27b	29	0
Actinoplanes sp. P15	29	2
Micromonospora sp. A401	33	1
S. griseus S86	1	1
S. vinaceus S208	12	3
N. corallina W5	14	1
M. phlei A400	6	0

*A total of 67 soil samples were studied, of these 27 contained 1 or more actinophages.

There seemed to be no prescribed pattern for actinophage distribution, with respect to the numbers and host ranges of phages isolated from individual soils. Soil samples from sites far removed from one another contained phages against a particular species. For example, a Minnesota soil sample and a Palos Blancos, Central America, soil sample both yielded lytic agents (MNP-6 and MNP-7, respectively) able to destroy *N. brasiliensis* N301. Likewise, *S. violaceoruber* S199 was attacked by phages MSP-15, 16, and 17 (isolated from Minnesota soil samples), by phage MSP-19 (isolated from Ohio soil), and by phage MSP-18, which was isolated from a Wisconsin soil sample. Apparently, the actinophage flora were determined by the type of soil (clay, loam, etc.) to a greater extent than by geographical location of the soil. Unfortunately, information about the sources of the soil samples used here was too incomplete to warrant an attempt to correlate soil type with viral flora. Normally, a phage attacking a particular actinomycete would be expected to be found in soil samples providing optimal conditions for growth of the presumed host. However, viruses able to lyse *N. brasiliensis* N301 were found in 11% of the soil samples collected in the United States of America even though this nocardia has been isolated only rarely here (Table 6). Actinophages having the power to lyse *S. griseus* strain S104 were detected in 12 of 16 soil samples; members of this group-species are readily cultivated from natural sources.

DISCUSSION

For the actinomycetes studied, kindredness as determined by viral susceptibility can be compared to that based on morphological characteristics such as color of soluble pigment, colony texture. arrangement of spores and color of the mycelia. Five *Streptomyces* strains (*S. cyaneus* S45, *S. vinaceus* S208, and *S. violaceoruber* strains S199, S16, and Sermonti 1) producing green, blue, or purple water-soluble pigments were closely related on the basis of susceptibility to actinophage. Also, 4 out of 5 of these strains exhibited spiraled arrangement of spores. Moreover, *S. olivaceus* S11 and the 7 strains of *S. griseus* employed in this investigation were similar morphologically, that is, all had straight spore chains and either produced brownish or no soluble pigment on the medium used. These 8 organisms formed a continuous related spectrum of susceptibility to actinophage. *Nocardia* sp. 3403 was the only soft, non-sporulating organism which could be included in the phylogenetic system based on susceptibility to actinophage; it was lysed by an actinophage that also attacked *S. griseus* S104. The other soft, nonsporulating organisms were susceptible to only 1 (or none) of the actinophages employed, and hence could not be compared phylogenetically on this basis to other strains.

It is interesting that no virus possessing the power to destroy *N. asteroides* N300 was isolated even though 24 attempts to obtain such a virus were made. It should be noted that *N. asteroides* can be isolated readily from soil samples. Perhaps the enrichment and detection media were inadequate or inhibitory for these actinophages. Contrariwise, phages virulent for *S. griseus* S104 were very common and could be isolated almost at will. The importance of the detection medium should be stressed. Phages MMP-2 and MMP-3, for example, were able to lyse *Actinoplanes* sp. P15 on peptone — yeast extract medium but not on a more complex medium which contained glucose, Difco casitone, and Difco beef in addition to peptone and yeast extract.

An analysis of cross-resistance patterns of selected virus-resistant actinomycetes can increase the resolution of phage susceptibility tests for taxonomic purposes. For example, *Streptomyces* strains selected for resistance to one actinophage become resistant to other actinophages frequently and rarely become sensitive to another phage. Mutants of related streptomycetes, selected for resistance to a particular virus, react similarly to other actinophages. In this way, the cross-resistance patterns of 12 streptomycete-phages of different origin and 7 strains of *Streptomyces* were compared, and then the organisms were arranged according to their biological affinities (Bradley, 1959a).

Although Welsch (1957) has isolated phages specific for certain streptomycetes, apparently monovalent phages are quite rare. Unless some mechanism not yet available can be used to restrict phages to a single species, susceptibility to actinophage cannot serve as a species characteristic. Strain-specific viruses, however, will be of no value in classification but may have limited application for identification. Contrariwise, the use of polyvalent phages in actinomycete taxonomy may be more promising. If the assumption is made that the genera *Streptomyces* and *Nocardia* are related so closely that they cannot be differentiated accurately using available taxonomic criteria (Bradley and Anderson, 1958), then all of the phages used in the present study, with one exception, were genus-specific. The susceptibility of *S. griseus* strain S104 to phage MMP-1, which was isolated on *Micromonospora* sp., represents the single exception. Phages MMP-2 and MMP-3, virulent for *Actinoplanes* sp., were specific for this organism. Therefore, it is possible that polyvalent phages can be employed in the identification of genera. Virus sensitivity alone cannot provide a complete taxonomy of the Actinomycetales; nevertheless, phage susceptibility studies should be of definite value in conjunction with improved morphological and physiological characters. Methodology for determining such taxonomic characters as color of mycelium, spore chain morphology, and production of soluble pigment needs to be reexamined and improved. In addition, characters such as antibiotic-synthesizing ability, antigenic-structure, spore arrangement and structure, carbohydrate utilization, nitrate reduction and hydrogen sulfide production have shown promise (Gottlieb, 1960). The use of actinophage susceptibility in conjunction with improved, accepted characters and newly introduced characters could provide new hope for actinomycete taxonomy.

The various organisms studied here can not be arranged linearly on the basis of susceptibility to actinophage; branching of the phylogenetic sequence may reflect inadequacy of the analysis or may provide a clue as to the origin of species. It is conceivable that rare hybridizations between a member of *S. violaceoruber* and a member of *S. griseus*, for example, yielded the forerunner of *S. vinaceus*. In all probability, gene recombination, as well as mutation and selection, has been a significant factor in the evolution of the enterobacteria (Ravin, 1960). In closing, it should be noted that the genera *Shigella, Salmonella*, and *Escherichia*, like *Nocardia* and *Streptomyces*, are difficult if not impossible to delineate. Perhaps they too should be placed in a single genus.

SUMMARY

Twenty-five strains representing 6 genera of Actinomycetales and 37 actinophages were employed in host range determinations. Five of 8 actinophages isolated originally on species of *Nocardia* were able to attack streptomycetes,

and 7 of 11 phages isolated against strains of *Streptomyces* lysed species of *Nocardia*. Strains of *S. violaceoruber* displayed similar susceptibilities to diverse actinophages. Conversely, *S. cyaneus* S45 and *S. griseus* S104 responded differently in most tests. Accordingly, the actinomycetes were arranged on the basis of corresponding viral susceptibility. Strains of *S. griseus* formed a continuous series whereas organisms designated *Nocardia* were apparently not connected directly with one another. Therefore, the genera *Nocardia* and *Streptomyces* were not delineated on the basis of virus sensitivity. *Mycobacterium phlei, Nocardia corallina, N. asteroides* N300, *Actinoplanes* sp. P15 and *Streptosporangium* sp. 27b could not be placed in the scheme presented. Actinophages could not be used in separating species but should prove useful in the identification of genera, group-species, and strains.

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A NEW BURIAL TECHNIQUE FOR IMPROVING THE SOIL BURIAL METHOD OF EVALUATING FABRIC PRESERVATIVE TREATMENTS

Vincent J. Bagdon and Samuel Shapiro

Fungus Control Section, Materials Branch, Technical Service Department
U.S. Army Engineer Research and Development Laboratories, Fort Belvoir, Virginia

The soil burial method is generally accepted as a very useful test for determining the effectiveness of fungitoxic treatments for cotton textiles. While this test was formerly considered by many investigators, particularly prior to 1943, to be rather unsatisfactory, it was strongly advocated and eventually adopted during World War II as the most practical means for predicting the field service performance of treated fabrics, viz., sandbag materials, tentage, etc., which required protection against continued soil contact (Bertolet, 1944; Dean *et al.*, 1945; Marsh *et al.*, 1945). Since that time, despite its shortcomings, the method has come more and more into general use throughout the world and its use has been extended as a drastic test for a realistic evaluation of all fabric preservative treatments. Much of its popularity is due to the simplicity of technique coupled with the fact that the method subjects the test fabrics under evaluation to immediate and simultaneous contact with a large and diverse population of soil microorganisms, among which there are always cellulolytic fungi and bacteria.

While there are many variations of the soil burial method, a classic example of this method is described in detail in Method 5762, "Mildew Resistance of Cloth; Soil Burial Method," of Federal Specification CCC-T-191, "Textile Test Methods." The conventional technique of burying fabric test specimens is to insert them vertically into the soil by means of a wide metal blade, usually called a "spud," shown in Fig. 1. This is accomplished by pressing one end of the specimen, at a point approximately 0.5 in. away from the end, down vertically into the soil with the spud until a little more than 1 in. of the specimen remains exposed above the surface of the soil. This burial technique is not very satisfactory, because maximum degradation may occur anywhere in the entire length of the buried fabric specimen. Consequently, in breaking strength determinations on specimens previously buried in this manner, quite often the break occurs in the jaws of the Scott Tester, or one end of the specimen is so rotted that it is impossible to determine the strength at the point of maximum degradation. This is graphically presented in Fig. 2A which depicts representative specimens after 18 days' burial in a vertical position.

In an attempt to resolve these deficiencies, an improved burial technique was developed wherein the center portion of the fabric specimen is buried in a "U-shaped" trench so that both ends of the specimen protrude approximately 1.5 in. above the surface of the soilbed. In this manner, the central 3-in. portion

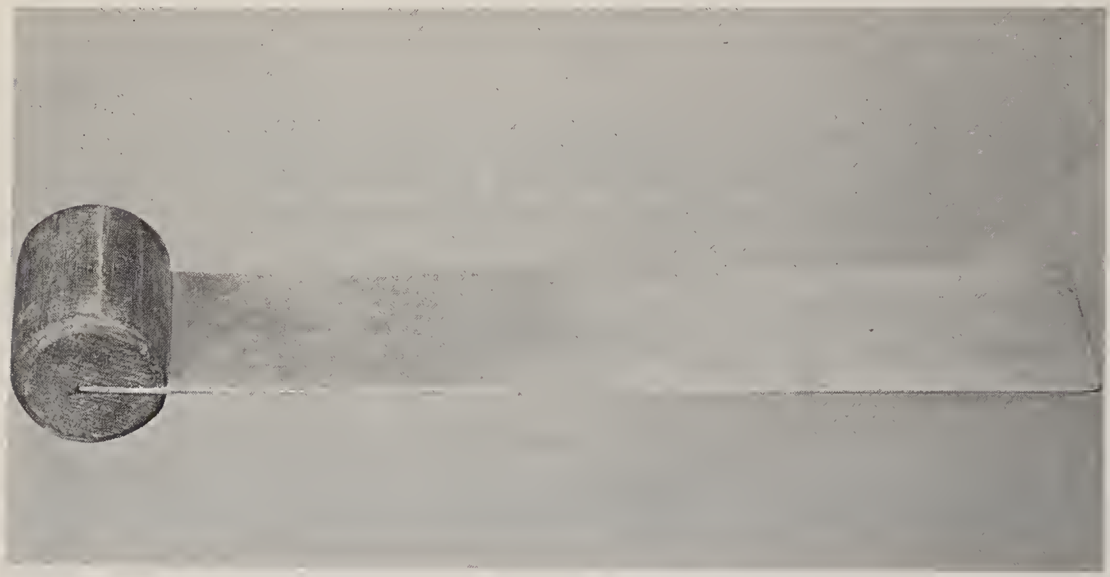

Fig. 1. Tool used for the vertical burial of fabric specimens.

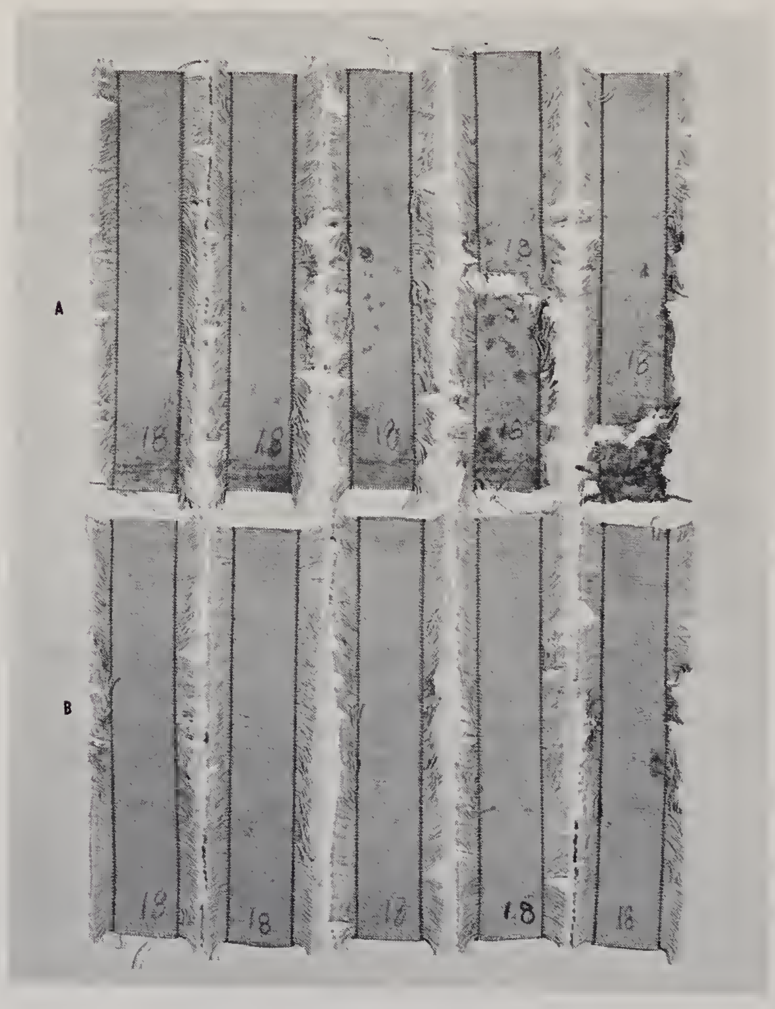

Fig. 2. Fabric specimens after 18 days of soil burial.
A) Buried by the vertical technique.
B) Buried by the "U-trench" technique.

of the specimen is buried an average of 1.25 in. below the surface and thus is spared the irregular gradation of rotting usually found along the length of a vertically buried fabric specimen. This insures that the maximum degradation will be in that 3-in. length which is between the jaws, when the specimen is broken in the Scott Tester. Figure 3 shows cotton-duck raveled specimens buried by this new technique; these specimens had been in the soil bed for several months when this photograph was taken. Grab method test specimens of jute and kenaf burlaps, 4 x 6 in. in size, buried by the same technique, are shown in Fig. 4.

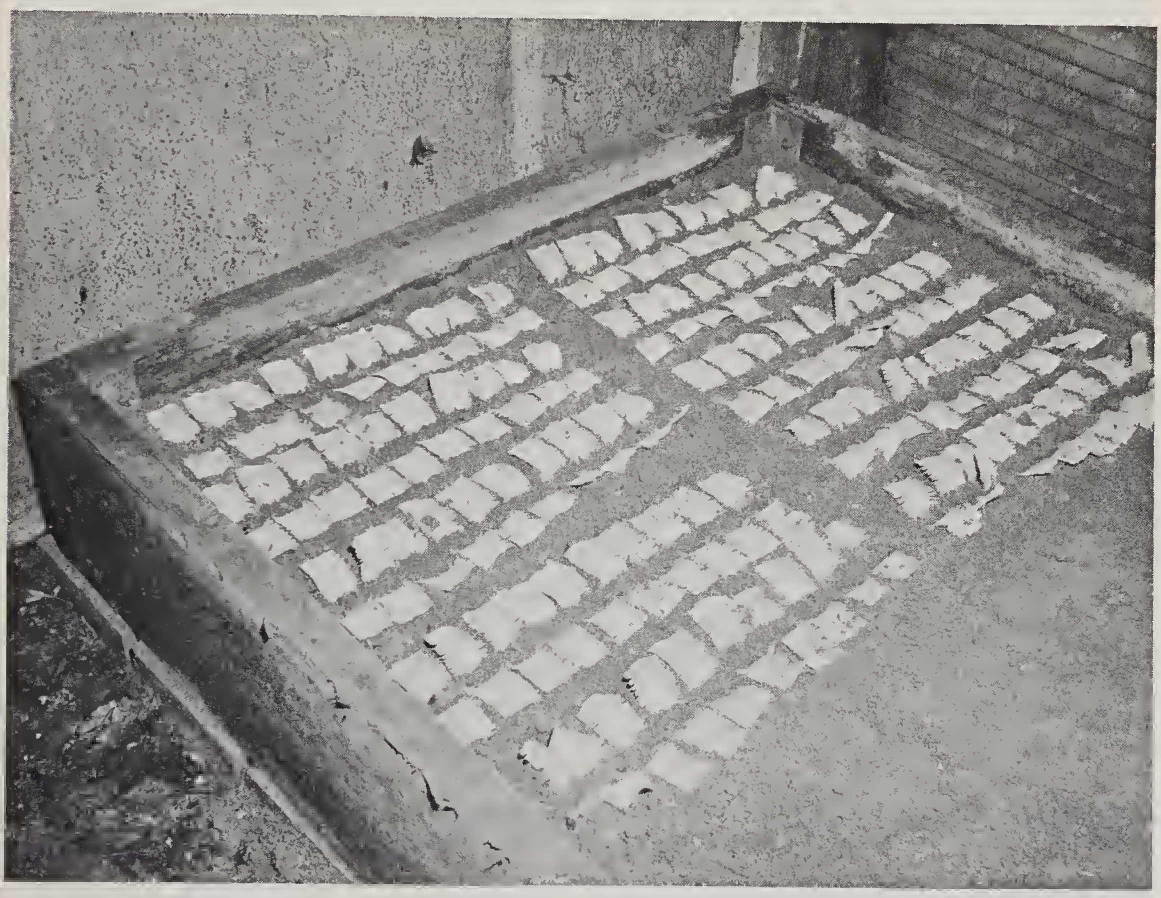

Fig. 3. 1-in. raveled specimens of cotton duck buried by the "U-trench" technique.

MATERIALS AND METHODS

The cotton duck used in this study was the standard "blue-line" fabric (Shapiro, 1957), which is used by many laboratories engaged in biodeterioration research. This fabric was impregnated with 2,2'-methylenebis (4-chlorophenol) at a concentration of 1.0% based on the dry weight of the fabric. Strips of this fabric were cut and raveled into test specimens which were randomized with the use of a random numbers table. These specimens were then divided into 7 sets of 20 each. Three sets each were buried by the previously described vertical and the "U-trench" techniques. The remaining set of specimens was retained in the laboratory for use as unexposed controls for original breaking strength determinations.

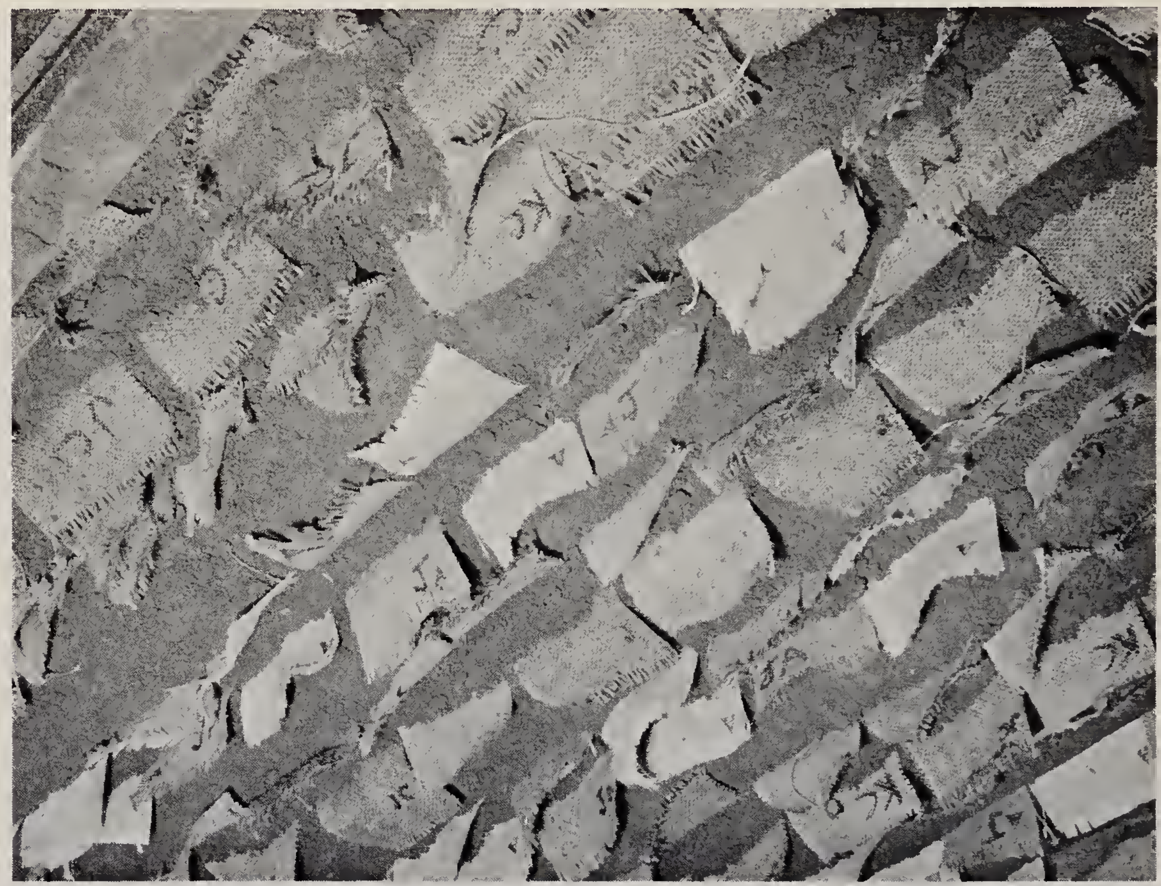

Fig. 4. 4 × 6-in. specimens of kenaf and jute burlaps buried by the "U-trench" technique.

In the "U-trench" burial technique, the surface of the soil bed was leveled and slightly compacted by the use of a flat metal plate. Then trenches were made with a specially designed trenching tool fabricated from 0.0625-in. stainless steel, as illustrated in Fig. 5A and B. Each end of the tool was slightly sharpened so that it would cut out a wedge of soil when it was pushed back and forth and at the same time would give the trench the proper contour. The wide flanges on either side limit the depth of the cut to approximately 1.25 in. and facilitate the preparation of a trench of uniform depth. If the walls of a trench were crumbly they were compacted and made firm by the use of a "trench-shaping" stick, illustrated in Fig. 5C, having the same contour but being slightly larger than the tool. The contour of the trench should be definitely in the shape of a "U" rather than a "V". It is quite possible that in a "V-shaped" trench the angle made by the sides would be sufficiently acute to prevent that portion of the specimen at the apex from being in direct contact with the soil. The fabric specimens were placed in the trench, as shown in Fig. 6, so that approximately 1.5 in. of each end of a specimen remained outside of the trench. Next, a little soil was sprinkled on the specimens to hold them in place, and then sufficient soil was used to fill the trench. The fill was finally compacted with the flat side of the "trench-shaping" stick.

The soil beds used in this comparative study are located in the Tropical Testing Chamber (Cooke and Vicklund, 1946) at the USAERDL, Fort Belvoir,

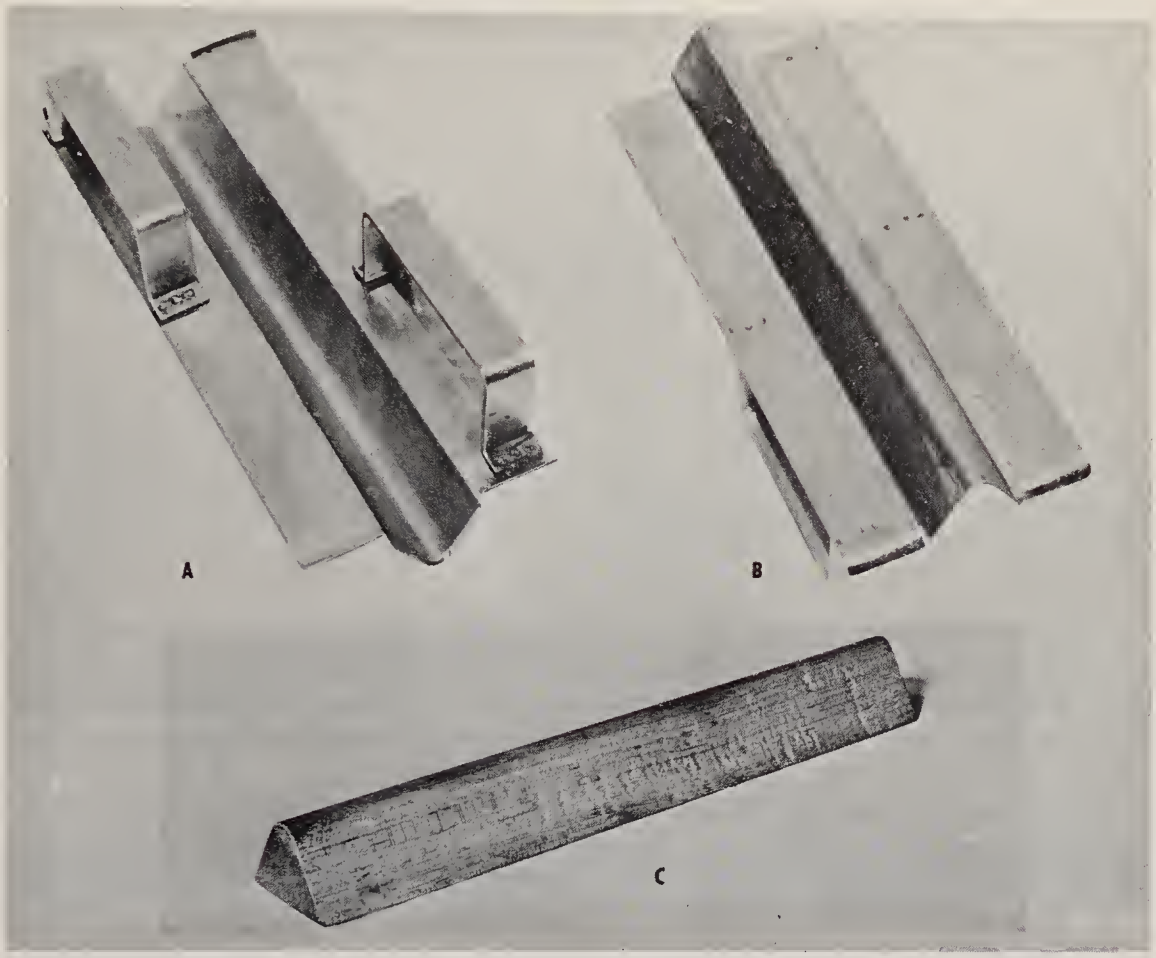

Fig. 5. Tools used for the "U-trench" burial of fabric specimens.
A) Top view of the trench tool showing flanges, handles, and the contour of the blade.
B) Bottom view of the trench tool.
C) Trench-shaping stick.

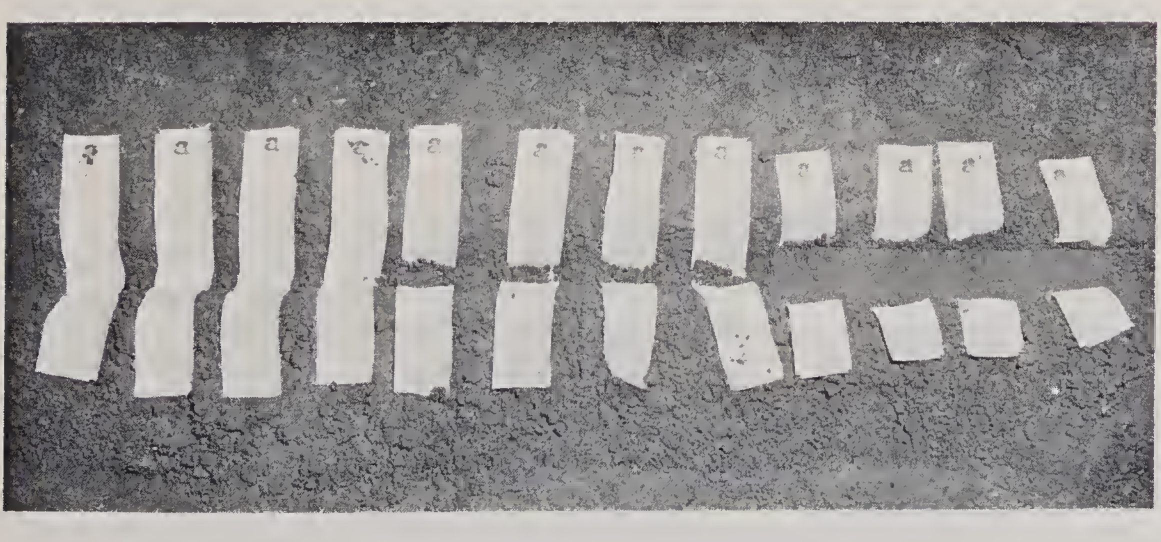

Fig. 6. Procedure for soil burial by the "U-trench"technique.
Left) Specimens placed in trench.
Center) Soil being replaced.
Right) Soil completely replaced and compacted.

Virginia. The temperature and humidity in the Chamber pass through a controlled cycle which simulates the daily cycling of these conditions in a tropical area. The use of this cycle has an advantage of restoring by condensation some of the moisture lost from the soil by evaporation. The soil in the beds was composed of a mixture of 2 parts of humus (leaf mold), 2 parts of sand, and 1 part of a natural sandy loam. These components were sifted through a 0.25-in. screen and then blended into a homogeneous mixture by the use of a concrete mixer. The resultant soil was porous and biologically active, with a moisture content between 24 and 28%, a pH between 6.4 and 6.9, and with little tendency to lump under hand pressure.

One set each of the fabric specimens buried by the two techniques was harvested after 10, 15, and 18 days of soil burial. The specimens removed from the soil bed were washed, dried, conditioned for 24 hr at 21.1 C ±1.1 C and 65% ±2% relative humidity, and then broken in a Scott Tester to determine the breaking strength lost during each burial period.

RESULTS AND DISCUSSION

Tables 1 and 2 show the mean breaking strength and the average strength loss, respectively, of the fabric buried by the two different techniques as compared with the mean breaking strength of the same but unburied fabric.

Table. 1. Mean Breaking Strengths (in lb) of Fabric Specimens Buried by the Two Techniques*

Type of burial	Days exposed in soil burial			
	0	10	15	18
Unburied (control)	101.1	–	–	–
Vertical	–	92.8	79.2	26.2
"U-trench"	–	94.4	84.3	60.8

*Each number represents an average for 20 replicate fabric specimens.

Table 2. Average Strength Loss (in %) of Fabric Specimens Buried by the Two Techniques*

Type of burial	Days exposed in soil burial		
	10	15	18
Vertical	8.4	21.8	74.1
"U-trench"	6.8	16.8	40.0

*Each number represents an average percentage of strength loss for 20 replicate fabric specimens, based on the original breaking strength of the same, but unburied, fabric.

Upon completion of the exposure periods, the following observations were made:

After 10 Days' Burial

Vertical technique. All specimens were beginning to show evidence of staining due to microbial attack. The mean breaking strength was 92.8 lb, with a standard deviation of 3.93. There was a loss of 8.4% of original breaking strength.

"U-trench" technique. The specimens buried by this technique showed little or no discoloration. The mean breaking strength was 94.4 lb, the standard deviation was 5.26, and the strength loss was 6.8%.

The degree of microbial degradation of the specimens buried by the two techniques was small and the difference was not significant.

After 15 Days' Burial

Vertical technique. Discoloration due to microbial degradation was more pronounced and diffuse on these specimens. The mean breaking strength decreased to 79.2 lb, the standard deviation was 19.01, and there was a strength loss of 21.8%.

"U-trench" technique. Staining became more evident on the central 3-in. portion of the fabric specimens. The mean breaking strength was 84.3 lb, with a standard deviation of 12.09. The specimens lost an average of 16.8% of their original breaking strength.

A comparison of the test data obtained on buried specimens using each of the burial techniques showed a greater amount of degradation for the specimens which were buried vertically. The standard deviation from the mean breaking strength was significantly smaller for those fabric specimens buried by the "U-trench" technique.

After 18 Days' Burial

Vertical technique. There was considerable variation in the general appearance of the buried portions of the fabric specimens. The evidence of biodeterioration ranged from slight discoloration to severe degradation wherein a specimen broke apart during removal from the soil bed (see Fig. 2A). The mean breaking strength was 26.2 lb, with a standard deviation of 19.82. There was a loss of 74.1% of original breaking strength. When the strength loss is as great as it is in this group of buried specimens, any statistical data obtained is considered to be of little value.

"U-trench" technique. There was a slight increase in discoloration due to microbial degradation over that observed on the specimens after 15 days of burial. All specimens were sound and intact, and were suitable for further testing (see Fig. 2B). The mean breaking strength was 60.8 lb, the standard deviation was 17.92, and the strength loss was 40.0%.

As shown in Fig. 2A, some of the specimens buried vertically were degraded to such an extent that it would have been impossible to determine the breaking strength of those specimens at the points of maximum degradation. The ends of all specimens buried by the "U-trench" technique were intact and all microbial damage was restricted to the central 3-in. areas on each specimen. Should it be desired, extremely degraded specimens can be easily removed intact from the soil because specimens buried by this technique are no deeper than 1.25 in. below the surface and are not subjected to the tension required to remove specimens which were buried vertically.

The "U-trench" burial technique has been utilized in our laboratory for the past few years in evaluations of fabric preservative treatments, and also in studies of the soil burial test method with a view to improving its reliability. This burial technique has been adopted for use in a proposed research procedure for the soil burial test, by an *ad hoc* working group sponsored by the Prevention of Deterioration Center, National Research Council, Washington, D. C.

SUMMARY

The conventional method of burying fabric test specimens in soil beds is not very satisfactory, because maximum degradation may occur anywhere in the entire length of the buried specimen. In breaking strength determinations of specimens buried by this technique, quite often the break occurs in the jaws of the Scott Tester, or one end of the specimen is so rotted that it is impossible to determine the breaking strength of the area of maximum degradation.

To obviate these shortcomings, a new burial technique has been developed. In this improved method, the fabric specimen is buried in a U-shaped trench with both ends of the specimen extending approximately 1.5 in. above the surface of the soil bed. This insures that the maximum degradation of the specimen will be in the 3-in. length which is placed between the jaws, when the buried specimen is broken in the Scott Tester.

Raveled specimens of standard "blue-line" treated test fabric were buried in soil beds using the two burial techniques. Sets of test specimens were removed from burial exposure at periodic intervals for examination and residual breaking strength determinations. The specimens which were buried by the "U-trench" technique were more uniformly degraded than those buried vertically. Microbial deterioration was confined to the central 3-in. portions of the former specimens, while those buried by the vertical technique showed irregular gradations of rotting along the entire length of the buried portions.

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USE OF VARYING CARBON SOURCES IN THE MEDIUM FOR THE DIFFERENTIAL ISOLATION OF STREPTOMYCES FROM SOILS

J.N. Porter and J.J. Wilhelm

Microbiology Department, Biochemical Research Section, Lederle Laboratories
American Cyanamid Company, Pearl River, New York

Although the great physiological variety of *Streptomyces* as a genus has created speciation difficulties which taxonomists have not yet fully resolved, it has proved to be an advantage to those seeking products of the group's metabolism. The predominance of different species and of forms within species may be manipulated by using environmental changes such as medium modifications, and thus it is possible to exploit this physiological variation. The present study is one of a series in which *Streptomyces* ratios in soil isolation plates have been altered by either subjecting soil samples to different conditions of incubation or by varying the conditions under which they may be plated out. This report concerns the possible use of carbon compounds in achieving the goal of differential isolation.

Three main points were investigated: 1) the ability of several *Streptomyces* species to form colonies when plated out on a basal medium supplemented with various carbon compounds; 2) the effect of the carbon source on isolations of some of these species from mixed culture; 3) the effect of the carbon source on isolations from nonsterile soils.

In order to test carbon utilization under conditions approximating those of isolation from nonsterile soil samples, a number of cultures, mainly representing described species, were established individually in soil which had previously been sterilized by autoclaving. The soil was pulverized to uniform particle size, placed in 20 g aliquots in 50 ml Erlenmeyer flasks and sterilized by autoclaving on 4 alternate days at 15 lb pressure for a minimum of 30 min each day. No contaminations were detected upon completion of this procedure.

For inoculation, mature yeast agar slant cultures were washed with sterile water, and 3 ml of spore suspension pipetted into each flask. The soil flasks were then incubated at 28 C for 3 weeks after which time a preliminary plating was carried out. During the incubation period the soils were moistened with sterile water at weekly intervals and at its completion the soil flasks were stored in a freezer at −15 C. Under this storage condition numbers did not change significantly over an 18-month period although 1 g samples were repeatedly removed from many of the flasks. Because a few cultures failed to become established under these conditions, the procedure of soil inoculation was later modified by scraping the surfaces of the slants into Hickey and Tresner's medium* and adding 7 to 10 ml of this suspension to each soil flask.

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*Difco yeast extract, 1.0 g; Difco beef extract, 1.0 g; N-Z amine "A," 2.0 g; amidex (Corn Products Refining Co.), 10 g; cobalt chloride heptahydrate, 20.0 mg; distilled water, 1000 ml; pH to 7.3 with sodium hydroxide.

The studies of Pridham and Gottlieb (1948) and of Benedict *et al.* (1955) have established that some carbon sources, such as starch, glycerol, or glucose, are apparently utilized by all *Streptomyces* species and that a few compounds are utilized by practically none. However, Benedict *et al.* reported on utilization patterns of 35 isolates representing 10 species in which certain carbon compounds supported growth of some of the cultures but not of all. Since such compounds could form the basis for the differential isolation of *Streptomyces* types it was decided to test a number of them as substrates. D(+) mannitol, L(+) rhamnose, lactose, i-inositol, sucrose, D(+) raffinose, L(+) arabinose, D(-) fructose, sodium citrate, salicin and D(+) xylose were chosen for this purpose. For comparison, development was also observed on glucose and, at times, on glycerol.

The synthetic basal medium of Pridham and Gottlieb* was used in all studies. Glycerol and salicin were added to this medium to give a 1% concentration prior to autoclaving. All other compounds were sterile-filtered and added to the autoclaved basal medium to give a 1% concentration with the exception of sodium citrate which was added at a rate to give a final 0.15% concentration.

One g of each soil culture to be tested was shaken with 10 ml of sterile water for 10 min on a Burrell wrist action shaker and then centrifuged at a slow rate of speed for 5 min in a table model International clinical centrifuge. Subsequent dilutions were made from the supernatant and mixed with the appropriate agars in Petri plates by the pour plate technique. Plates were incubated at 28 C for 1 week, at the end of which time observations on the degree of colonial development were made.

Eighteen cultures representing 14 described species were studied. Three of the cultures did not belong to known species. Two cultures were included of *Streptomyces griseus*, 1 a streptomycin and 1 a viomycin producer. As shown in Table 1, 12 of the 18 strains developed fully on mannitol, 8 on rhamnose, 5 on lactose, 7 on inositol, 1 on sucrose, 2 on raffinose, 7 on arabinose, 11 on fructose, 2 on sodium citrate, and 6 on salicin. Six of the cultures were tested on xylose, 2 producing mature colonies. None of the cultures tested on glycerol or dextrose failed to display normal development.

It is evident that a variety of patterns of colony development occurred among the strains tested. In the extreme cases, *S. purpurascens* developed well on all substrates with the exception of sodium citrate while *S. griseocarneus* showed no development, although plating on other media showed it to be present and viable in the soil flask. Among the other strains a variety of responses are to be noted.

It seemed of interest to compare the behavior of our strains with that of the strains tested by Benedict *et al.* (1955) as listed in their publication. Table 2 shows such a comparison for *S. griseus*, *S. lavendulae* and *S. purpurascens*, the only species tested in common. Substantially good agreement was achieved.

Since the purpose of investigating the various carbon substrates was to be able to isolate some strains preferentially from mixed populations, a number of the cultures tested individually as described were mixed in pairs and plated out on selected carbon sources. Aqueous dilutions were prepared from the soil flasks

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*$(NH_4)_2SO_4$, 2.64 g; KH_2PO_4, 2.38 g; K_2HPO_4, 5.65 g; $MgSO_4 \cdot 7H_2O$, 1.00 g; $CuSO_4 \cdot 5H_2O$, 0.0064 g; $FeSO_4 \cdot 7H_2O$, 0.0011 g; $MnCl_2 \cdot 4H_2O$, 0.0079 g; $ZnSO_4 \cdot 7H_2O$, 0.0015 g; agar, 15 g; distilled water, 1000 ml; pH 6.8 to 7.0 before autoclaving.

Table 1. *Streptomyces* Colony Development on Various Carbon Sources

Streptomyces Culture Designation		Carbon Sources													
		D(+)Mannitol	L(+)Rhamnose	Lactose	i-Inositol	Sucrose	D(+)Raffinose	L(+)Arabinose	D(-)Fructose	Sodium citrate	Salicin	Glycerol	D(+)Xylose	Dextrose	P & G Basal
A-1	*Streptomyces griseus* (streptomycin producer)	+	-	-	-	-	-	±	+	+	+	+	±	+	-
A-20	*S. cinnamomeus*	+	+	±	±	±	±	±	+	+	+				±
A-377	*S. aureofaciens*	-	-	-	-	±	-	±	±	-	-	+	±	+	-
A-1155	*Streptomyces* sp.	+	+	+	+	±	±	+	+	±	+				±
A-3160	*S. lavendulae*	-	-	-	-	-	-	-	-	±	±				-
A-3343	*S. netropsis*	-	-	-	±	-	-	-	-	±	-				-
A-7575	*S. violaceoruber*	+	+	+	±	-	±	+	±	±	±	+	+	+	-
A-8846	*S. rimosus*	+	-	±	+	-	±	±	+	-	-	+	-	+	-
A-9665	*S. griseus* var. *purpureus* (viomycin producer)	+	-	-	-	-	-	-	+	-	-	+	-	+	-
AA-289	*Streptomyces* sp.	+	+	±	+	-	±	+	+	±	-				-
AA-931	*S. tanashiensis*	-	-	±	-	-	-	±	-	±	+				-
AC-578	*S. griseocarneus*	-	-	-	-	-	-	-	-	-	-				-
AC-889	*S. hygroscopicus*	+	±	±	-	-	-	+	+	-	±				-
AD-120	*S. purpurascens*	+	+	+	+	+	+	+	+	±	+	+	+	+	-
AD-316	*S. felleus*	-	+	+	-	-	-	±	-	±	+				-
AD-592	*Streptomyces* sp.	+	+	±	+	-	-	+	+	±	-				-
AD-702	*S. antibioticus*	+	+	+	+	-	-	±	+	±	-				-
AE-206	*S. fulvissimus*	+	-	-	+	-	+	+	+	±	-				-

+: Full development. ±: Partial development. -: No or very poor development.

Table 2. Colony Development by Three Species of *Streptomyces* on Various Carbon Sources as Compared with the Utilization Pattern of Benedict *et al.* (1955)

Carbon Source	*S. griseus*			*S. lavendulae*		*S. purpurascens*	
	A-1 Streptomycin producer	A-9665 Viomycin producer	Benedict *et al.* 8 strains	A-3160	Benedict *et al.* 7 strains	AD-120	Benedict *et al.* 3 strains
D(+)Mannitol	+	+	+(8)	-	-(7)	+	+(3)
L(+)Rhamnose	-	-	+(3) -(5)	-	-(7)	+	+(3)
Lactose	-	-	+(7) -(1)	-	-(7)	+	+(3)
i-Inositol	-	-	+(1) -(7)	-	-(7)	+	+(3)
Sucrose	-	-	+(1) -(7)	-	-(7)	+	+(3)
D(+)Raffinose	-	-	+(2) -(6)	-	-(7)	+	+(3)
L(+)Arabinose	±	-	+(4) -(4)	-	-(7)	+	+(3)
D(-)Fructose	+	+	+(8)	-	-(7)	+	+(3)
Salicin	+	-	+(8)	±	+(4) -(3)	+	+(3)
D(+)Xylose	±	-	+(8)	No test	-(7)	+	+(3)

+: Good growth. ±: Partial growth. -: Growth very poor or none.

in such a way as to provide approximately equal numbers of colonies on developed agar plates. Table 3 shows the result of plating seven such pairs and that it is possible to manipulate the carbon source so that one member of the pair, but not the other, will produce isolatable colonies. Deep colonies, which are difficult or impossible to assess in mixed culture, can be eliminated by the use of a surface layering technique. However, cleancut separation was not achieved in some mixtures tested because of partial development by the weaker member of the pair, possibly stimulated by the presence of the other.

Table 3. Colony Production by Individual Members of Paired Cultures of Streptomycetes on Various Carbon Sources

			Mannitol	Rhamnose	Lactose	Fructose
1.	A-1	*S. griseus*	+	-	-	+
	AD-316	*S. felleus*	-	+	+	-
			Inositol	Salicin		
2.	A-1	*S. griseus*	-	+		
	A-8846	*S. rimosus*	+	-		
			Fructose	Salicin		
3.	A-8846	*S. rimosus*	+	-		
	AA-931	*S. tanashiensis*	-	+		
			Inositol	Salicin		
4.	A-1	*S. griseus*	-	+		
	AA-289	*Streptomyces* sp.	+	-		
			Mannitol	Salicin		
5.	A-9665	*S. griseus* var. *purpureus*	+	-		
	AA-931	*S. tanashiensis*	-	+		
			Mannitol	Rhamnose	Lactose	
6.	A-9665	*S. griseus* var. *purpureus*	+	-	-	
	AD-316	*S. felleus*	-	+	+	
			Mannitol	Rhamnose	Inositol	Salicin
7.	AA-289	*Streptomyces* sp.	+	+	+	-
	AA-931	*S. tanashiensis*	-	-	-	+

+: Good colony formation. -: Colonies not observed.

Platings of nonsterile soils were carried out to see if the predominant culture types on isolation plates would be altered by providing different carbon substrates. The Pridham and Gottlieb basal medium was used and the carbon compounds were selected from the same group used to test the development of the individual cultures.

It was noted that colonial development was generally inferior on plates containing lactose, sucrose or sodium citrate. The distribution of *Streptomyces* types from a greenhouse soil is shown in Table 4 as an example of the type of result obtained. Colonies representing only the predominant types were picked from each plating and the isolates were compared on three media to determine the cross-identity between them. Each culture type was then designated by a letter and the distribution of the 33 types isolated from plates containing 12 carbon sources is shown in the table.

Table 4. Distribution of Common Types Isolated from a Greenhouse Soil on the Pridham and Gottlieb Basal Medium Fortified with Different Carbon Sources

Carbon Source	Distribution of Common Types	Total	No. Occurring on the One C Source Only
Glucose	A - B - C - D - E - F - G	7	2
D(+)Mannitol	A - B - D - H - I - J	6	0
L(+)Rhamnose	A - B - H - K - L - M	6	2
Lactose	A - B - D - H - J - N - O - P	8	2
i-Inositol	A - C - H - P - Q - R	6	1
Sucrose	A - D - H - J - S	5	1
D(+)Raffinose	A - C - D - H - I - T - U	7	2
L(+)Arabinose	A - B - C - D - H - M - V	7	1
D(-)Fructose	A - B - D - H - M - W - X - Y - Z	9	3
Sodium citrate	D - E - H - R - AA - AB - AC	7	3
Salicin	A - B - H - J - AD - AE - AF	7	2
Glycerol	A - B - D - E - H - Y - AE - AG	8	1

The number of common types picked from each set of plates ranged from 5 to 9. The most frequently occurring types throughout were A and H, which occurred on 11 of the 12 carbon sources. Types B and D occurred on 8 sources but not 4, and the other types were isolated less frequently. Thus, it appears that changes in populations on isolation plates may result from the use of different carbon substrates.

While the object of a soil isolation program in screening for industrial products is to provide microbial cultures capable of varied physiological potentials, it is worthwhile to supplement routine screening programs with searches for less predominant organisms or those with uncommon growth requirements. Among the various ways to achieve this is the use of unusual substrates for isolation purposes. The demonstration of varied carbon utilization patterns indicates the potential usefulness of different carbon substrates in isolating the unusual among the streptomycetes.

SUMMARY

A number of carbon compounds known to support the growth of some streptomycetes but not all were incorporated in the Pridham and Gottlieb basal medium, and cultures previously established in sterile soil plated out on the various medium modifications. The patterns of colony formation varied with the strain and

the carbon source. The preferential isolation of cultures from mixtures was achieved in some instances. The predominance of various cultures isolated from a nonsterile soil was shown to change with the carbon sources.

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THE EFFECT ON STREPTOMYCES POPULATIONS OF ADDING VARIOUS SUPPLEMENTS TO SOIL SAMPLES

J.N. Porter and J.J. Wilhelm

Microbiology Department, Biochemical Research Section, Lederle Laboratories
American Cyanamid Company, Pearl River, New York

By virtue of their role in antibiotic production and various microbiological transformations, streptomycetes have been the subject of many isolation programs. Traditionally these have taken the form of extensive isolations from soils collected in a wide variety of geographical locations. Although numerous investigators have attempted to correlate *Streptomyces* populations with habitat, little has been reported on the intensive study of relatively few soil samples. Furthermore, those making ecological studies have usually failed to report a breakdown of their isolates as to strains or species, preferring to record their data as total numbers of actinomycetes in populations of other microorganisms.

We have studied the effect of adding various supplements to soil samples to ascertain their effects on numbers of *Streptomyces;* and in the case of certain additives, such as cottonseed meal and dried blood flour, more intensive experiments have been carried out in which the *Streptomyces* populations have been analyzed qualitatively. This paper is concerned with the results of a number of our observations in these respects.

Initially, we were interested in finding out whether soil enrichments would have any effect at all on streptomycete numbers and, if so, what types of additives were most effective in this respect. Consequently, a number of materials which were readily at hand were added individually to soils collected in a random manner for the soil isolation program. The supplements, mostly organic, were added at the arbitrarily chosen rate of 15 mg/g of soil. The soils were weighed out in 5 g quantities and placed in sterile 50 ml Erlenmeyer flasks, moistened with from 1 to 2 ml of sterile water depending on the water-holding capacity of the sample, and incubated at 28 C for 2 weeks.

It soon became evident that molds and bacteria would become a major problem and that a change in isolation procedure would be necessary in order to isolate *Streptomyces* preferentially. This was satisfactorily achieved by utilizing the Benedict modification of the Lindenbein medium supplemented with the antifungal antibiotic pimaricin. The details of this method are given in a recent publication (Porter *et al.*,1960). All isolations, except where indicated otherwise, were made with this medium combination. For isolating *Streptomyces*, 1 g of soil was suspended in 10 ml of sterile water, shaken for 10 min on a Burrell wrist action shaker and then centrifuged at a slow rate of speed for 5 min in a table model International clinical centrifuge. Subsequent dilutions were made from the supernatant.

Soils have been supplemented with over 50 substances. Table 1 shows the results of a number of these, selected as being representative of the types of compounds tested. Because of the wide divergence of *Streptomyces* counts it was felt that the median value was a truer indication of the type of result obtained than was the mean. Counts were greatest from materials with high nitrogen content, the various crude meals being particularly good in this respect. The simpler nitrogen compounds or those containing no nitrogen were relatively ineffective, at least under the conditions tested. Although it is not readily apparent from the table, it was repeatedly observed that the increase in numbers of streptomycetes was accompanied by less variety in the final dilution plates, an indication that the major increase in numbers was restricted to certain types.

Table 1. Effect of Various Additives on the Isolation of *Streptomyces* from Soils

Additive	No. of Soil Samples Tested	No. of Streptomycetes per g of Soil (median value in millions)	Average No. of Cultures Isolated Per Sample	% of Samples with Heavy Bacterial Populations
Salmon viscera meal	45	2736.0	11	29
Peanut meal	63	955.2	11	27
Distillers' solubles	40	739.1	11	35
Cottonseed meal	97	373.9	14	22
Linseed meal	37	200.0	14	19
Soybean hydrolysate	40	120.0	12	48
Dried blood flour	78	82.4	14	31
Yeast extract	57	77.6	12	42
Dried brewers yeast	73	25.4	13	38
Corn starch	40	8.4	12	5
Ammonium sulfate	16	8.2	13	6
Sodium citrate	52	5.1	12	31
Lactose	18	3.1	12	11
Xylose	27	2.4	13	33
Urea	43	1.4	12	5
Dry, no additive	100	0.8	14	3
Moist, no additive	100	0.3	15	8

Thus, it became evident that many organic substances containing nitrogen were capable of producing sizable increases in numbers of streptomycetes. (It must be kept in mind, however, that because of the adherence of spores in chains, counts in soil samples are only approximate in cases where streptomycetes have been stimulated to produce new sporophores.) It next seemed desirable to investigate the effect of one or two additives in more detail and to assess the effect on the predominant or isolatable strains or types.

Consequently, 400 g of a greenhouse soil were pulverized in a mortar and distributed in 50 g aliquots in 8 500-ml Erlenmeyer flasks. Four soil treatments were involved, with duplicate flasks for each treatment. Table 2 shows the 4 incubation conditions employed. Isolations were made at 14, 21, and 28 days from the start of the experiment by the method previously described. Fifty streptomycete colonies were picked from each set of plates representing a soil flask, a total of 400 per isolation time or 1200 in all. No attempt was made to isolate all possible strains from these plates since the basic purpose was to see if a shift in predominant types occurred after treatment.

Table 2. Effect on *Streptomyces* Population of Adding Cottonseed Meal to a Greenhouse Soil

Incubation Time (days)	Incubation Conditions	Average No. of Streptomycetes per g of soil	Average No. of Types Isolated (out of 50)
14	Dry, −4C	3.8×10^6	21
	Dry, + 28C	2.95×10^6	18
	Moist, + 28C	3.8×10^5	19
	Moist, CM, + 28C	4.25×10^6	14
21	Dry, −4C	1.1×10^6	18
	Dry, + 28C	1×10^7	18
	Moist, + 28C	8.1×10^7	22
	Moist, CM, + 28C	2.3×10^{11}	9
28	Dry, −4C	2.2×10^6	15
	Dry, + 28C	2.1×10^6	19
	Moist, + 28C	1.6×10^6	21
	Moist, CM, + 28C	3.0×10^{13}	13

Although the classification of streptomycetes is difficult because of the extreme variability of the group, results could not be surveyed qualitatively until the isolates had been typed by morphological and physiological criteria. Development, sporophore morphology, spore color, soluble pigments, surface texture, and reverse colors were observed on yeast, Benedict, Czapek, and Hickey and Tresner's cobalt amidex media. Together with the ability to darken iron peptone agar these criteria permitted the arrangement of all isolates into classes or types. Certain supplementary observations, such as antibiotic activity, were also frequently used. There was no attempt to make species determinations of the isolates.

Of the theoretical total of 1200 cultures, 57 failed to grow or were contaminated. The remaining 1143 cultures represented 136 types as determined by the

Table 3. Distribution of Three *Streptomyces* Types In Greenhouse Soil Treated with Cottonseed Meal

Soil Treatment	*Streptomyces* Type	Number of Times Isolated*		
		14 Days	21 Days	28 Days
Dry, −4C	Q	30	22	44
	AE	1	0	1
	BK	0	1	1
Dry, + 28C	Q	29	26	23
	AE	0	0	0
	BK	0	0	1
Moist, + 28C	Q	24	23	12
	AE	1	7	3
	BK	1	4	0
Moist, CM, + 28C	Q	4	0	0
	AE	32	43	27
	BK	25	18	29

*Out of a total of 100 isolates.

Table 4. Effect on *Streptomyces* Populations of Adding Cottonseed Meal or Dried Blood Flour to a Greenhouse Soil

Incubation Time (Days)	Incubation Conditions	Isolation Medium	No. of Streptomycetes per g of Soil	No. of Types Isolated (out of 25)
14	Dry, −4C	B	1.7×10^7	12
		ADA	1.2×10^7	13
	Moist, + 28C	B	4×10^5	15
		ADA	2.7×10^5	10
	Moist, CM, + 28C	B	1.4×10^9	7
		ADA	1.4×10^9	7
	Moist, DBF, + 28C	B	4.7×10^7	3
		ADA	4.4×10^7	4
21	Dry, −4C	B	2.9×10^6	10
		ADA	4.4×10^5	8
	Moist, + 28C	B	1.9×10^9	18
		ADA	1.4×10^8	10
	Moist, CM, + 28C	B	2.0×10^9	6
		ADA	1.8×10^9	6
	Moist, DBF, + 28C	B	1.0×10^{11}	14
		ADA	2.3×10^{10}	*

B: Benedict agar.
ADA: Asparagine dextrose agar.
*Heavy bacterial contaminations made isolations impossible.

above criteria. Table 2 shows an increase in numbers of several thousandfold upon treatment with cottonseed meal plus moisture. However, under the same condition there was a decrease of the isolatable types of about one half. The shift of numbers of the predominant types, three of which are shown in Table 3, is of interest. Type Q, the most common, was isolated 237 times in the entire experiment but only 4 were from flasks treated with cottonseed meal. Types AE and BK behaved in the reverse fashion, the former being isolated 102 times out of 115 from the cottonseed meal flasks and the latter 72 times out of 80.

Table 5. Effect on *Streptomyces* Populations of Adding Cottonseed Meal or Dried Blood Flour to a Field Loam (Collected at Harrison, Indiana)

Incubation Time (Days)	Incubation Conditions	Isolation Medium	No. of Streptomycetes per g of Soil	No. of Types Isolated (out of 25)
14	Dry, -4C	B	2.9×10^7	21
		ADA	2.5×10^7	14
	Moist, + 28C	B	2.8×10^7	22
		ADA	2.5×10^7	15
	Moist, CM, + 28C	B	6.9×10^9	14
		ADA	7.3×10^9	13
	Moist, DBF, + 28C	B	3.7×10^9	5
		ADA	*	
21	Dry, -4C	B	7.3×10^6	17
		ADA	7.8×10^6	24
	Moist, + 28C	B	3.0×10^7	20
		ADA	1.4×10^8	18
	Moist, CM, + 28C	B	3.4×10^{12}	17
		ADA	1.7×10^{13}	13
	Moist, DBF, + 28C	B	5.4×10^{10}	13
		ADA	*	

B: Benedict agar.
ADA: Asparagine dextrose agar.
*Heavy bacterial contaminations made isolations impossible.

A second experiment of this type was run, in which two additives, namely, cottonseed meal and dried blood flour, and three soils were employed. Duplicate flasks were not used and only 25 cultures were isolated from each flask at two isolation dates, 14 and 21 days. Two isolation media, Benedict and asparagine dextrose agars, were used. The amount of supplement (15 mg/g), moisture, incubation conditions, isolation procedure and classification of types were the same as previously described.

The results shown in Tables 4, 5, and 6 agree with those from the previous experiment. Numbers are generally increased in the presence of both cottonseed meal and dried blood flour but the variety decreases. The variety of isolates was not reduced by the use of asparagine dextrose agar but bacterial contaminations were heavier, preventing *Streptomyces* isolation in some cases.

Table 6. Effect on *Streptomyces* Populations of Adding Cottonseed Meal or Dried Blood Flour to a Field Loam (Collected at Goshen, Indiana)

Incubation Time (Days)	Incubation Conditions	Isolation Medium	No. of Streptomycetes per g of Soil	No. of Types Isolated (out of 25)
14	Dry, -4C	B	1.6×10^6	18
		ADA	1.1×10^6	20
	Moist, + 28C	B	1.6×10^8	15
		ADA	1.3×10^8	17
	Moist, CM, +28C	B	2.7×10^9	5
		ADA	1.5×10^8	11
	Moist, DBF, + 28C	B	3.4×10^7	3
		ADA	1.9×10^7	9
21	Dry, -4C	B	4.9×10^6	24
		ADA	3.7×10^6	19
	Moist, + 28C	B	1.4×10^8	20
		ADA	1.1×10^8	21
	Moist, CM, + 28C	B	1.3×10^{10}	19
		ADA	2.0×10^9	13
	Moist, DBF, + 28C	B	1.5×10^{11}	15
		ADA	*	

B: Benedict agar.
ADA: Asparagine dextrose agar.
*Heavy bacterial contaminations made isolations impossible.

Table 7 gives the distribution of the 17 most commonly occurring types of the 328 isolated in the experiment. Their distribution is influenced by both the soil sample and treatment of the sample. Type A, for example, occurred predominantly in greenhouse soil and over 50% of the 118 isolates were obtained from flasks treated with cottonseed meal. Type BT, on the other hand, was isolated only from Harrison soil treated with dried blood flour.

The results of these experiments show, therefore, that both the numbers and predominant types in *Streptomyces* populations can be influenced by soil enrichment techniques. The implication cannot be ignored that rarer types, which may be overlooked by standard isolation methods, may become isolatable following soil additive supplementation.

Table 7. Distribution of 17 Common *Streptomyces* Types in Soils Supplemented with Cottonseed Meal or Dried Blood Flour

Soils	Occurrence of Common Types																
	A	B	C	E	J	O	V	W	AC	AF	BK	BP	BR	BT	BZ	CQ	FO
Greenhouse	100	10	11	11	24	9	35	4	10	1	0	4	0	0	0	1	18
Harrison	1	5	1	3	9	3	0	6	9	11	13	6	12	18	1	0	6
Goshen	17	23	2	4	3	2	15	2	25	2	0	13	0	0	15	14	2
Incubation Condition																	
Dry, -4C	32	15	5	4	20	5	0	5	1	5	2	3	2	0	9	0	0
Moist, +28C	17	11	8	1	5	6	1	4	1	5	1	6	0	0	7	1	5
Moist, CM, +28C	68	10	0	4	10	1	0	0	0	3	8	7	10	0	0	14	21
Moist, DBF, +28C	1	2	1	9	1	2	49	3	42	1	2	7	0	18	0	0	0

SUMMARY

Soil enrichment techniques may strongly influence the numbers and distribution of *Streptomyces* types in soil populations. Complex nitrogenous materials appear particularly useful in this respect. Cottonseed meal and dried blood flour, examples of such additives, produced increases in *Streptomyces* numbers and a shift in predominant types but yielded less variety on isolation plates than nontreated soils.

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STRUCTURE AND ANTIFUNGAL ACTIVITY OF THIOLSULFONATES AND RELATED COMPOUNDS

(ABSTRACT)

S. S. Block, T. P. Johnston, and W. H. C. Rueggeberg

University of Florida, Gainesville, Florida,
Southern Research Institute, Birmingham, Alabama, and
Atlas Powder Co., Wilmington, Delaware.

A series of phenyl thiolsulfonates and compounds of related structure were examined for antifungal activity. Observations of the data suggest the following relationships between structure and activity.

(1) The $-SCCl_3$ group appears to be a toxiphore. In most cases it greatly increased the toxicity over that which might have been expected from a similar structure without this group. Furthermore, in no case tested did it reduce to any degree the toxicity of a similar structure without the group.

(2) Although it does not demonstrate the high activity of the $-SCCl_3$ group, the thiolsulfonate group, $-SO_2S-$, appears to be a toxiphore. Most compounds containing the $-SO_2S-$ structure were fungitoxic. Simple sulfides and disulfides without the $-SO_2-$ linkage lacked activity.

(3) Substitution of hydrogen atoms for chlorine in $-SCCl_3$ reduces toxicity markedly but does not eliminate it.

(4) A comparison of compounds having RSO_2SCCl_3 and RSO_2CCl_3 shows the necessity of the thiolsulfonate sulfur for toxicity.

The data suggest the possibility that the ultimate toxiphore is the thiol sulfur (-S-) and that the $-CCl_3$ in $-SCCl_3$ as well as the $-SO_2-$ and -N- in RSO_2SCCl_3 and $RNSCCl_3$ act as intensifiers of the toxicity.

PSEUDOMONAS AERUGINOSA AS A MATERIALS BIODETERIORATION TEST ORGANISM

Walter M. Bejuki

Prevention of Deterioration Center, National Research Council, Washington, D.C.

INTRODUCTION

Recently a more formal recognition of bacterial degradation of various materials has been evidenced in the incorporation of various bacterial forms into microbiological assay procedures for the purpose of determining at the laboratory level, the significance and characteristics associated with bacterial influence on materials other than food and drugs. Specifically several published methods have appeared in ASTM procedures (Standard Methods, 1958 a, b, c); several more informal, exploratory or research efforts are under consideration. Although microbiological deterioration of organic materials has been primarily associated with the fungi, some degree of unrest has been expressed by numerous microbiologists who have felt that bacterial deterioration should be given a more formal contemporary evaluation. The significance of bacterial decay of material, based on economic considerations is as yet an undetermined quantity on most substrates. The obvious possible exceptions would seem to be iron corrosion and slime formation in paper pulp by bacteria. In organic materials such as cotton, wool, plastics, leather, and paper the microbiologist has been preoccupied primarily by the fungi. Siu indicates in his review of 1951 that possibly this preoccupation is due to two reasons: one, the generally more easily observed, more obvious infestation by fungi made this appear to be primarily a fungus problem and as such, attracted men trained particularly in mycology. Secondly, the more delicate nature of the bacteria did not permit transhipment and survival of bacterial cultures when, during the early days of cellulolysis evaluations, samplings were being made throughout the tropics, far from ideal laboratory facilities. However, many bacteria were isolated and Ralph Siu's book very adequately catalogs these in terms of their ability to break down cellulose-containing materials. The more omnivorous predilections of bacteria for various substrates has been observed by many microbiologists before and since and the table is presented as only an indicative sample.

A rogues' gallery such as this could be expanded by hundreds of species. Reviews have been made by Levinson (1948), Office of Scientific Research and Development through Reuszer (Alabama Agricultural Experiment Station, 1945), Stahl (1943), Harris (1959) and others. The enigma indicated here seems to be that with this fairly diverse broad interest the bacterial significance in microbiological deterioration continues to be without definitive or broad support. This however is not our subject for discussion today.

Table 1. Bacteria Associated with Degradation

Organism	Substrate	Reference
1. *Clostridia*	cellulose	Janke (1949)
2. *Micromonospora propionici*		
3. *Desulfovibrio desulfuricans*	ceramic clays	Johnson *et al.* (1949)
4. *Bacillus pandora*	latex	Simonart (1952)
5. *Micrococcus epimetheus*		
6. *Erwinia nimipressuralis*		
7. *Bacillus mesentericus*	wool	Nopitsch (1952)
8. *B. subtilis*		
9. *B. megaterium*		
10. *B. mycoides*		
11. *B. prodigiosum*		
12. *Pseudomonas aeruginosa*	vinyl films	Daoust *et al.* (1951)
13. *Mycobacterium* sp.	natural and synthetic rubber	Zobell & Beckwith (1944)
14. *Pseudomonas*		
15. *Corynebacterium*	a) 4,6-dinitro-o-cresol b) p-nitrophenol c) 2,4-dinitrophenol d) 2,4,6-trinitrophenol (picric acid)	Jensen & Gundersen (1955)
16. *Vibrio desulfuricans*	iron	Anon. (1947)
17. *Sporovibrio desulfuricans*	iron	Starkey (1945)
18. Bacteria	plywood glues	Christensen (1945)
19. *Thiobacillus concretivorus*	concrete	Parker (1947) Taylor & Hutchinson (1947)

At the Prevention of Deterioration Center we are very much interested in microbiological test procedures and have been approached on occasion for advice on personnel hazards associated with this type of bioassay. Our studies indicate that the microfungi used in the various specifications and standards have been adequately reviewed for safety, and suspect organisms removed. This is a continuing process and currently *Myrothecium verrucaria,* a possible test fungus, is being evaluated for its potential health hazard. Earlier, *Aspergillus fumigatus,* introduced as a test organism many years ago, was removed because of its known pathogenic property. The bacteria however are microorganisms being newly introduced into biodeterioration evaluations, and the information background and hazard evaluation has not been as thoroughly reviewed. Our case in point today is *Pseudomonas aeruginosa.* Although a test organism for bioassay purposes must have many qualities such as a sound taxonomy, be stable in form, physiology, and biochemistry, easily cultured, adaptable to laboratory conditions, and not fastidious, in our industrial microbiological laboratories, the factor of personnel safety must be given a high priority. Unlike the pathology lab where highly qualified, specialized personnel predominate, the industrial test laboratory called upon to meet microbiological specification requirements will often be staffed with fine engineers, chemists, and physicists who have little or no training in the biological sciences. Even with biologically trained personnel a nonpathogenic organism can be handled with greater economy of equipment, care, and time, so that apart from safety considerations a nonpathogen should be the preferred test organism. Our Center was invited to comment on the proposed use of *P. aeruginosa* as a bacterial test organism. This organism is currently being considered as a test bacterium in the study of microbial alteration of the characteristics, both structural and aesthetic, of various plastic material in sheet, cast, and extruded forms. It is generally agreed that the basic resins have a high degree of resistivity, and vulnerability is associated with the plasticizer added to give the various forms desirable properties, primarily flexibility and impact resistance.

Current readings in the bacterial literature indicate that *Pseudomonas aeruginosa* is becoming an organism of greater concern in medical clinics and clinical laboratories (Davis *et al.*, 1959). There is some evidence in the modification of intestinal biota that the use of penicillin and possibly other antibiotics is creating new conditions conducive to the increase in number and importance of *P. aeruginosa,* a penicillin-resistant bacterium. Clifton (1950) states quite clearly "It [*P. aeruginosa*] is becoming of more importance at the present time; since penicillin therapy may overcome the common gram-positive cocci also present, the balance established between these species is upset, and the gram-negative, penicillin resistant *P. aeruginosa* can continue to develop."

Current interest in this organism clinically is revealed in four representative papers, which I will cite by title and source only (Gaby and Hadley, 1957; McDonald *et al.*, 1958; Mitchell *et al.*, 1951; Orr *et al.*, 1958).

In view of this evidence indicating that *P. aeruginosa* is possibly an unsafe choice of organism for assay purpose in industrial microbiology where trained personnel will not always be available, a further look at this organism is in order.

PSEUDOMONAS IN RETROSPECT

A brief review of the biochemistry, enzymology, pathology, and variability of this organism indicates that we have here a ubiquitous and versatile organism, a facultative anaerobe, many potentialities of which we are just becoming more aware. Gale (1947) and Stephenson (1949) have much to contribute on the biochemistry and enzymology of the Pseudomonaceae, and Dubos (1946) cites the work of Legroux and Genevray, 1933, on converting an avirulent, colorless, and nonproteolytic culture of *B. pyocyaneus* into a virulent and pathogenic form, by two consecutive passages in the culture filtrate of a virulent, pigmented, and proteolytic strain of the same species.

A beautiful picture of this organism is presented by Zinsser and Bayne-Jones (1937):

> Among the pigment-producing organisms, there is one group which contains at least one member whose pathogenic capacity is established. Medical bacteriologists have been accustomed to refer to these organisms (motile, gram negative, producers of fluorescent green, yellowish, and blue pigments) as members of the *pyocyaneus* group taking the name from the long-known species, *Bacillus pyocyaneus*. Organisms with these characteristics are widely distributed in nature, being found in water, soil, and intestinal contents of man and animals. The genus, which is therefore of general interest, has been finally set apart by Bergey under the designation of *Pseudomonas* and the medically important species has been named *Pseudomonas aeruginosa*, adopting the nomenclature of Schröter and Migula.
>
> . . . .
>
> [It was] first demonstrated by Gessard in 1882. The bacillus which was described by Gessard has since become the subject of much careful research and has been shown to hold a not unimportant place among pathogenic bacteria.
>
> *B. pyocyaneus* is one of the less virulent pathogenic bacteria.

It is found frequently as a harmless parasite; however, occasionally it has been found

1) in connection with suppurative lesions of various parts of the body,
2) in chronic otitis media in ill-nourished children,
3) in stools of children suffering from diarrhea,
4) throughout organs of children dead of gastro-enteritis,
5) in spleen after general sepsis following a mastoid operation,
6) during life in pericardial exudate,
7) in pus from liver abscesses,
8) in umbilical infections in newborn children.

> Similar examples of *B. pyocyaneus* infection in human beings might be enumerated in large numbers, and there is no good reason to doubt, that, under given conditions, fatal infections may occur.
>
> In guinea pigs intraperitoneal inoculation with a loopful of a culture of average virulence usually leads to the death of a young guinea pig within three or four days.

Topley and Wilson (1936) have this to add: "It may invade nasal fossae, the middle ear, the meninges, the bronchi and other organs, and set up suppuration. According to Pons (1927) *Ps. pyocyaneus* is especially pathogenic in the tropics where it not infrequently is responsible for typhoid-like infections and abscess of the liver."

These references indicate fairly well the classical attitude toward the pathology of this organism.

PSEUDOMONAS TODAY

Although it is readily conceded that *Pseudomonas tabaci* would be primarily of interest to the plant pathologist, at this time and for our purpose it is well to note that E. D. Garber (1959) reports on *Pseudomonas tabaci* by saying, "Preliminary studies on biochemical mutants of this pathogen indicated that . . . certain biochemical mutants exhibited a pattern of virulence and avirulence for susceptible species of *Nicotiana* and varieties of *N. tabacum*." In this last statement I would emphasize "a pattern of virulence and avirulence."

Correspondence with men actively studying *Pseudomonas* spp. in their current research brings opinions such as the following. Allow me to begin with the most recent letter. This is dated July 22, 1960 and comes from Dr. E. C. S. Chan (1960) of the Canada Department of Agriculture, Research Branch, Microbiology Research Institute, Ottawa. He says, "You are correct in saying that *Pseudomonas aeruginosa* is too pathologic for an industrial lab. Although it was once assumed to be a harmless saprophyte and only a pathogenic opportunist with slight pathological power, it is today incriminated as sole etiological agent of endocarditis and pneumonia."

Another communication, dated June 23, 1960, comes from Professor H. M. Tsuchiya (1960), University of Minnesota. "I share your apprehension about the use of *Pseudomonas aeruginosa* as a bioassay test organism in deterioration of plastics. Indeed even organisms such as *Escherichia coli* are pathogenic under certain conditions. Hence I would take a stronger position than you in stipulating that biologically trained personnel work with any and all microorganisms." Dr. Tsuchiya states that he has been interested in biological depolymerization and polymerization for the past twelve years.

My next quotation too is from a personal communication received in early March of this year from Irving Davis, Captain, United States Air Force, and Walter Sellers, Medical Bacteriologist, both stationed at that time at the School of Aviation Medicine, USAF Aerospace Medical Center (ATC), Brooks Air Force Base, Texas (Davis and Sellers, 1960). Their reply reflects also the opinion of two colleagues with whom they consulted.

From paragraph 2:

> Although *Pseudomonas aeruginosa* is essentially an opportunist rather than an obligate pathogen, we agree with you [that you] would be well advised to reconsider the use of this bacterial species. A few lines of evidence as to the pathogenicity of *Pseudomonas aeruginosa* may be stated. The Surgical Research Unit, Brooke Army Medical Center, San Antonio, possibly receives more burn patients than any other continental hospital. Bacteriologists at this installation inform us that this organism, *Pseudomonas aeruginosa*, in the past few years has supplanted the staphylococci in numbers of infections developing in burn cases. As a generality if this organism is involved in a general septicemia, death of the patient may be anticipated. This has been the experience at Brooke Army Medical Center as well as other hospitals. Another point of importance is that this organism does apparently develop complete resistance to many of our common antibiotics. In fact, each of our stock culture strains utilized in our research and obtained from hospital infections are resistant to several of the antibiotics and, indeed, some of them are not susceptible to any antibiotic that we have on our shelves.

In paragraph 3 the following information is reported:

> A few years ago our Microbiology Cellular Biology Branch monitored a research contract with the Florida State Health Department laboratories. These investigators over a period of several years examined more than 7000 individuals for the predominating organisms associated with otitis externa infections. *Pseudomonas aeruginosa*, often obtained in pure culture, was the principal organism in over three quarters of their clinically diagnosed otitis externa cases.

From paragraph 4:

> However, the techniques (in utilizing *Pseudomonas aeruginosa)* that would have to be developed to permit the aerosolization of this microorganism and its bioassay, as well as to maintain the maximum degree of safety for the personnel, would be so stringent as to preclude its practical use.

In paragraph 5, we are reminded that Bergey's manual, Edition VII, lists approximately 150 *Pseudomonas* descriptions. Of these only two are pathogenic to humans: *P. aeruginosa* and *P. pseudomallei.* This suggests exploration and possible utilization of some of the other pseudomonad species.

In visit to PDC offices in August of 1960, Dr. James T. Sinski reported that in his screening for bacterial susceptibility to crystallized fire ant venom at Tulane University, *Pseudomonas aeruginosa* was the only bacterium of 30 aerobic forms tested (including 10-15 strains of staphylococcus) that was not affected by the venom. This venom has so far been chemically characterized only as an amine.

Dr. Sinski also noted that the Drs. Earl and Thelma Fisher are currently searching for therapeutants to control *P. aeruginosa* eye infections. This is an additional example of a directly vulnerable organ quite liable to infection as the ordinary industrial microbiological test methods for materials susceptibility now stand.

In seeking further information on the background, origin, and opinion at the source from which the proposed *Pseudomonas aeruginosa,* further identified as ATCC No. 13388 and this equivalent to QMB-1468, came, I visited with and discussed this bacterium with Dr. Hillel Levinson at the Quartermaster Corps, Natick, Massachusetts on March 3, 1960.

Tracing back the history of this particular organism, Dr. Levinson was able to indicate that this was a strain of what was formerly known as *Bacillus pyocyaneus,* isolated from pus at Wyeth laboratories by Maurice Landy and was at that time also identified as Culture No. 60 of the Wyeth group. It was principally used in phenol coefficient testing. It was not the isolate associated with depletion of plasticizer in vinyl-coated rain gear. We discussed the wisdom of using this strain for any assay, and although we recognized the ubiquitous nature of this organism we also recognized that it does not occur in high population counts suspended in test tubes and neither is it in nature dispersed as an aerosol in such an extremely high count, as any test method would require. We felt that if a suitable substitute could not be found a strong warning on pathogenicity and a screening evaluation for the pathology of this particular strain would be the least concession that should be allowed in permitting the use of this organism in any proposed method.

We consulted Dr. Arthur Kaplan, who suggested we stay away from the pus former, substitute the raincoat isolate, if such exists, and modify the test so that aerosolization is not necessary. Vincent Bagdon of Engineers Research and Development Laboratories at Belvoir indicates that a reevaluation is in order.

A few well managed, well staffed federal and industrial test laboratories are currently using this organism in their plastics deterioration studies. Apparently no ill effects have resulted. The *P. aeruginosa* being used was presumed to be a raincoat isolate and has proven to be a tractable laboratory test species. Whether a facultative parasite need continue to be used is debatable. Where pseudomonads have been associated with materials deterioration, often the species identity has been incomplete. Possibly more research in this area will provide us with an obligate saprophyte which will be a more suitable test organism.

CONCLUSIONS

1) Bacteria as test organisms in industrial microbiology, reflected in specifications testing, represent a fairly new group of bioassay organisms, and their use by both biologically trained and untrained individuals is associated with new control and evaluation problems.

2) Penicillin and other antibiotic therapy has led to new conditions conducive to increasing the significance, number, and importance of *Pseudomonas aeruginosa,* a penicillin-resistant bacterium.

3) A random sample of studies involving *Pseudomonas aeruginosa* indicates that here, as in several other bacterial species, physiological strains, specific resistivity, and mutations not reflected in cell morphology, are creating a complex, biochemical, biophysiological-strain, identification problem.

4) Strains have been conditioned from avirulent, nonpigmented, nonproteolytic forms to virulent, pigmented, and proteolytic forms of the same species. Other parameters such as lysis, involution, dissociation, etc., have been shown to be unstable.

5) Classic references to significant pathologic potential indicate a long history of association of this organism with suppurative lesions, chronic otitis media, diarrhea, gastroenteritis, umbilical infections, liver abscesses, septic spleen, endocarditis, external otitis, and other diseases.

6) Recent medical studies indicate that resistant strains of *Pseudomonas aeruginosa* are posing, in some fields, e.g. burn therapy, control problems as significant as those associated with resistant strains of staphylococcus.

SUMMARY

I should like to close by indicating that I believe it is historically the correct time to introduce, intelligently, thoughtfully, and with deliberate convictions, the use of bacteria as test organisms in assaying the microbiological deterioration of many industrial materials, including plastics. I recognize that in considering the problems in relation to health hazards, significance of the test, reliability, mutation potential, technique, and similar factors, profound care and infinite harvesting and utilization of known facts will have to be exercised. I have every confidence however that the fine work initiated by and reflected in test methods of the ASTM and other industrial and Federal groups will grow into a fine collection of productive and safe assays suitable for use in the industrial microbiological laboratory.

In the meantime it is probably well to recognize that in some areas of materials deterioration bacteriology we are practicing scientific brinkmanship. Brinkmanship, as I understand it, either in diplomacy or science, is hazardous.

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RESISTANCE OF ORGANIC MATERIALS TO MARINE BACTERIAL ATTACK

Priscilla L. Steinberg

Bell Telephone Laboratories, Inc., Murray Hill, New Jersey

With the advent of submarine cables for transoceanic telephone communication the Bell System has become increasingly interested in the marine environment. One aspect of particular interest in this environment concerns the effect that the biological population may have on materials used in cable construction. Considerable efforts have been made to gather fundamental data concerning the resistance of applicable organic materials to attack by marine microorganisms.

A test program was originated in 1954 to assemble both accelerated laboratory test data for engineering use and also data on actual behavior of materials in the ocean. Figure 1 is an outline of the complete marine biological test program as it currently exists. There are two laboratory tests, the first of which is designed to measure the resistance of organic materials to aerobic and anaerobic microorganisms. This paper is concerned with describing the conduct of this test and data obtained from it. In the second test, the conductance and capacitance of wire samples coated with a ten mil thickness of the organic material are measured periodically and used as an indication of their performance in a sea water and sediment medium.

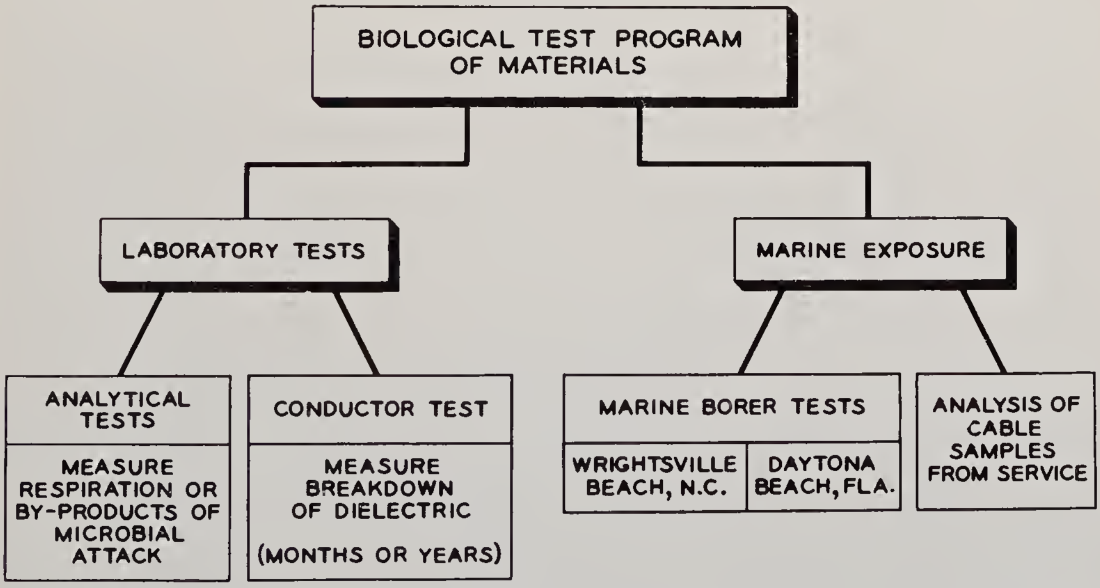

BIOLOGICAL TEST PROGRAM OF MATERIALS
LABORATORY TESTS
MARINE EXPOSURE
ANALYTICAL TESTS
MEASURE RESPIRATION OR BY-PRODUCTS OF MICROBIAL ATTACK
CONDUCTOR TEST
MEASURE BREAKDOWN OF DIELECTRIC
(MONTHS OR YEARS)
MARINE BORER TESTS
WRIGHTSVILLE BEACH, N.C.
DAYTONA BEACH, FLA.
ANALYSIS OF CABLE SAMPLES FROM SERVICE

Fig. 1. Outline of marine biological test program.

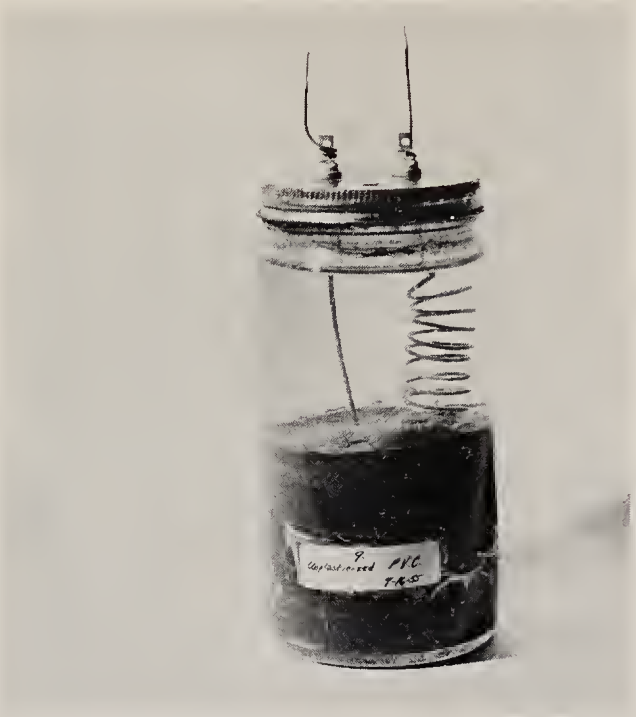


Fig. 2. General setup of conductor test showing a coil half in sediment and half in sea water.

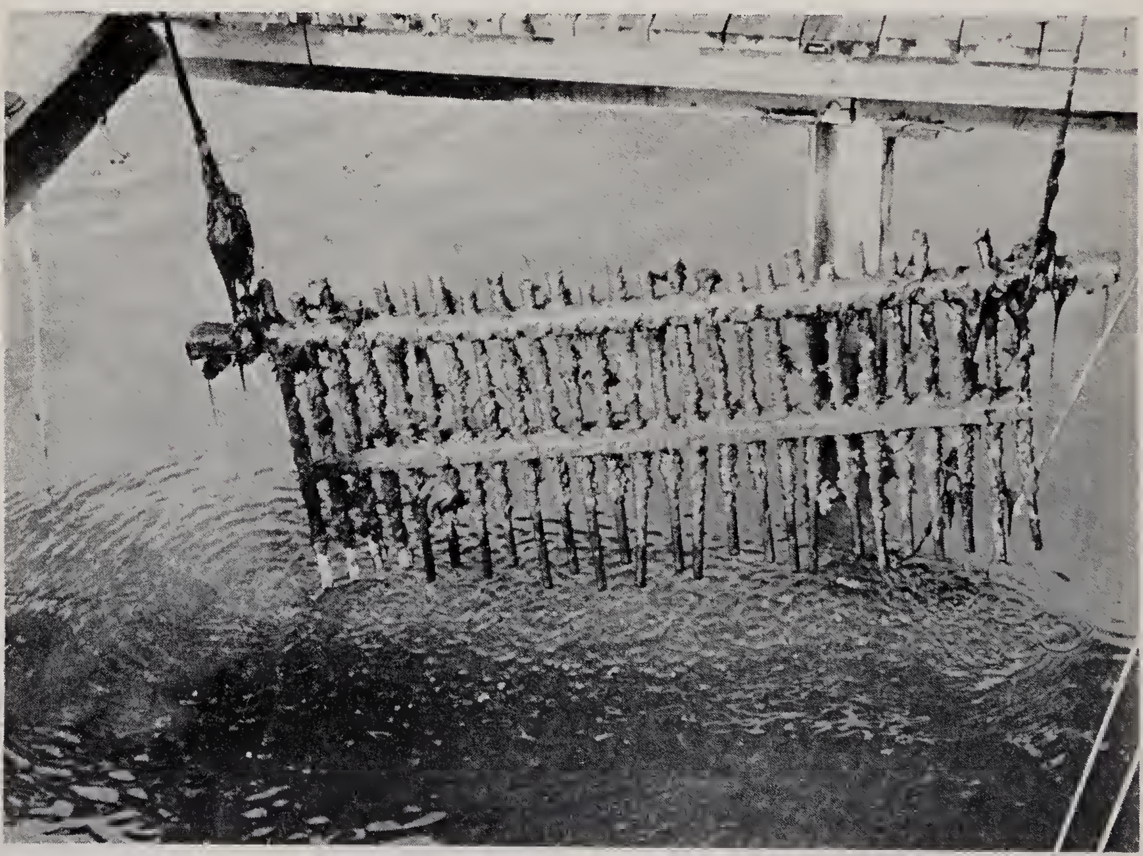


Fig. 3. Rack being removed from natural exposure test location for yearly examination.

A marine exposure test program was included primarily to determine the extent of marine borer and also microbiological attack in the natural sea water and sediment. In cooperation with the William F. Clapp Laboratories, Inc. two test areas on the Atlantic coast have been equipped with racks containing a wide variety of organic samples. For this test the materials are supplied in rod form whenever possible, and those available only as fibers or tapes are wound on lucite rods and attached to the racks. The racks are equipped with an untreated wood bait strip which is placed across the midsection of the test specimens. This piece functions as a pathway for the marine borers to travel from the water to the material. Where the bait piece is not in contact with the samples it is possible to determine whether the organisms can attack directly from the water. The samples are to be exposed indefinitely but are removed for yearly inspection.

The second area of the natural exposure program is concerned with the examination of sections of submarine cables returned from service. In the examination of these cables any defects in insulating materials, corrosion, fungal degradation of the wrappings, or evident changes in the physical appearance of the cables are studied. Ocean bottom sediment samples are requested from the area in which the cable section is removed. The sediment samples obtained are analyzed in an effort to relate the chemistry of the environment to the performance of cable materials and also to add more information to the inadequate data available on the composition of the ocean floor.

BIOCHEMICAL OXYGEN DEMAND TEST

For the purpose of the laboratory testing program Biochemical Oxygen Demand (BOD) may be defined as the amount of dissolved oxygen in parts per million consumed by aerobic bacteria during an 8-week incubation of the organic materials in aged and inoculated sea water. Since the test samples supply the only available source of carbon for the marine bacteria, the rate of oxygen consumption is a direct measure of the utilization of the material.

This test program has provided a method of screening materials as to their effectiveness in a marine environment. However, since it is an accelerated laboratory test the data obtained must be evaluated accordingly. Possible changes in the materials due to prolonged exposure cannot be foreseen from these data, yet such changes could alter the degree of resistance of the material to marine bacteria. Therefore, the purpose of conducting this test is to supply basic and immediate data on the ability of the materials as they exist to function as a carbon source for marine bacteria. Other laboratory tests and studies must be relied on to complete the data on the effectiveness of the materials.

The BOD test itself is conducted as seen in the flow chart in Fig. 4 using aged sea water which has been saturated with oxygen. At least 8 weeks prior to running the test, sea water and ocean bottom sediment are collected from a carefully selected area on the Atlantic coast. Some of the material to be tested is added to a portion of the collected sea water and sediment and the mixture is inoculated from pooled enrichment cultures. These enrichment cultures are made up for each test sample and incubated at 25 C for at least 6 weeks prior to testing. The purpose of preparing separate cultures is to enable microorganisms capable of utilizing the various materials to develop preferentially under ideal conditions.

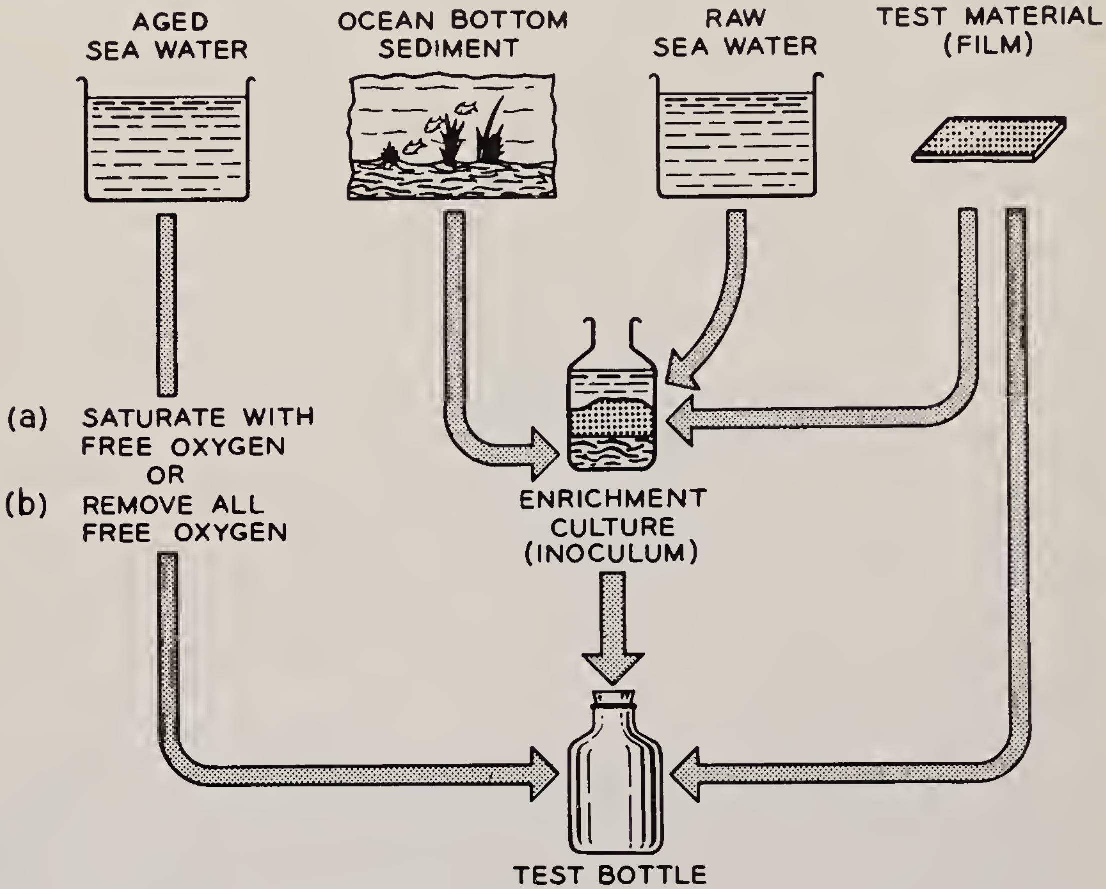

AGED SEA WATER
OCEAN BOTTOM SEDIMENT
RAW SEA WATER
TEST MATERIAL (FILM)
(a) SATURATE WITH FREE OXYGEN
OR
(b) REMOVE ALL FREE OXYGEN
ENRICHMENT CULTURE (INOCULUM)
TEST BOTTLE

Fig. 4. Flow chart of Laboratory Bacteriological Test.

In the meantime, the sea water to be used is filtered and aged in the dark until the organic content is less than 1 ppm.

Incubation of the samples is conducted in 60 ml glass-stoppered bottles, allowing 10 bottles for each test sample. This supplies sufficient samples to measure the dissolved oxygen content of the water following 0, 1, 2, 4, and 8 weeks of incubation in a water bath. However, when a high rate of attack is anticipated, the incubation intervals are shortened in order to obtain more titration data. Two replicate samples are sacrificed for titration after each interval. Usually 6 materials are included in each test run and one set of control bottles suffice per run; thus, approximately 70 bottles are placed in test simultaneously.

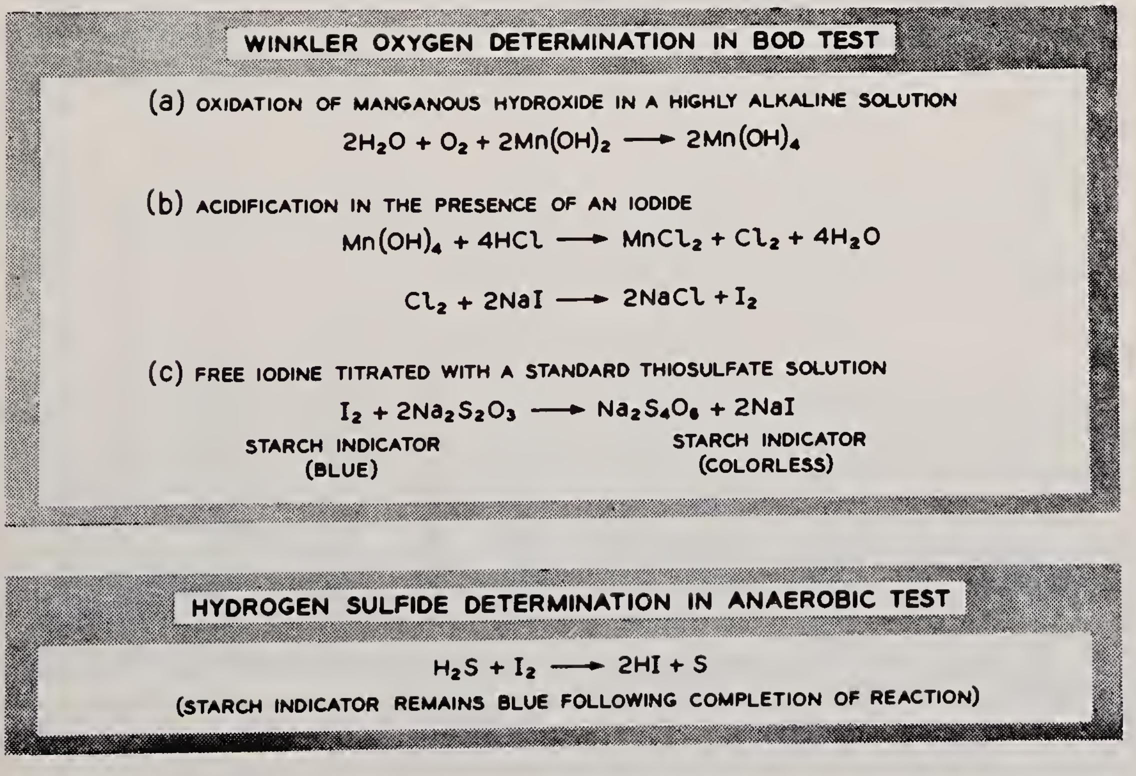
Whenever possible, the samples are obtained in sheet form about 4 mils thick in order to subject equal surface areas to attack. Each bottle contains a 1- in.^{2} sample but when only granular material is available, 0.05 g is used.

When these preliminary procedures have been carried out, the aged sea water is placed in a carboy and aerated with oxygen for 16 hrs. A combined enrichment culture containing a few drops from each of the incubated cultures of the sample group being tested is added to the aerated sea water in a concentration of 1 ml per 10 L of sea water. This concentration of inoculum provides maximum microbial activity with the least amount of added organic material.

A slight oxygen pressure is placed on the water until after the test bottles are filled, thus assuring oxygen saturation throughout the period prior to incubation.

The incubation of the test samples has been carried out in water baths held at two different temperatures, namely, at 20 C and 5 C. When the test was originated, the 20 C temperature control was chosen as the lowest temperature from the range which could support maximum microbial activity. However, as will be noted in the data, the utilization of oxygen at that temperature was so rapid in some cases, that the dissolved oxygen was completely consumed by the end of 1 week of incubation and it was difficult to screen the materials as to relative performance. Due to this rapid consumption of oxygen and also to the fact that the Bell System is primarily interested in the intensity of attack at ocean bottom conditions, a second series of BOD tests was run using 5 C as the water bath incubation temperature. Since the cooler temperature reduced the rate of microbial activity the incubation period was extended over a longer duration of time and provided a better opportunity for comparing the resistances of the various materials to attack.

The dissolved oxygen content before incubation and following the different incubation periods is measured using a modified Winkler method. The basic Winkler procedure consists of three steps as shown in Fig. 5: (1) oxidation of



WINKLER OXYGEN DETERMINATION IN BOD TEST

(a) OXIDATION OF MANGANOUS HYDROXIDE IN A HIGHLY ALKALINE SOLUTION

$$2H_2O + O_2 + 2Mn(OH)_2 \longrightarrow 2Mn(OH)_4$$

(b) ACIDIFICATION IN THE PRESENCE OF AN IODIDE

$$Mn(OH)_4 + 4HCl \longrightarrow MnCl_2 + Cl_2 + 4H_2O$$

$$Cl_2 + 2NaI \longrightarrow 2NaCl + I_2$$

(c) FREE IODINE TITRATED WITH A STANDARD THIOSULFATE SOLUTION

$$I_2 + 2Na_2S_2O_3 \longrightarrow Na_2S_4O_6 + 2NaI$$

STARCH INDICATOR (BLUE) — STARCH INDICATOR (COLORLESS)

HYDROGEN SULFIDE DETERMINATION IN ANAEROBIC TEST

$$H_2S + I_2 \longrightarrow 2HI + S$$

(STARCH INDICATOR REMAINS BLUE FOLLOWING COMPLETION OF REACTION)

Fig. 5. Reactions involved in laboratory marine analytical tests.

manganous hydroxide in a highly alkaline solution, (2) acidification of the product in the presence of an iodide, and (3) volumetric titration of the liberated iodine with a standard thiosulfate solution using a starch indicator.

The oxygen consumed is calculated from the results of the titrations of the sea water prior to incubation and following the longest period of incubation. The difference between these two values over and above any loss of oxygen in the control samples constitutes the BOD of the test material.

ANAEROBIC TEST

The anaerobic test procedure employed in the laboratory involves the measurement of hydrogen sulfide produced as a by-product during the utilization of the organic materials by anaerobic sulfate-reducing bacteria. Since sulfate-reducing bacteria are ubiquitous, the determination of their activity is indicative of the extent of anaerobic attack.

As indicated in the laboratory test program flow chart, the preparations for the test are quite similar to those for the BOD test with the exception of the handling of the aged sea water. In the anaerobic test the sea water is boiled vigorously for 10 min and siphoned immediately into a carboy. A slight nitrogen pressure is placed on the carboy until the water is cooled enough to add the inoculum. Additional nitrogen pressure is kept on the carboy while the bottles are filled in order to prevent free exchange of oxygen with the water. Since the anaerobes function much more slowly than the aerobic bacteria the incubation periods selected are 0, 4, 8, 12 and 16 weeks at 20 C. In general, the rate of attack at 20 C is very slow, and it was assumed that very little microbial activity, if any, would be detected if the incubation were carried out at 5 C. Therefore, only 20 C data are presented on the utilization of the organic materials by anaerobic bacteria.

Following incubation the hydrogen sulfide dissolved in the sea water is titrated with an iodine solution using starch as an indicator. Hydrogen sulfide content calculated over and above that found in the inoculated controls is considered a measure of anaerobic attack of the material in test.

DISCUSSION OF COMBINED DATA

In discussing the results of this test program the word "attack" is used quite freely when describing the utilization of organic materials by the bacteria. The word "attack" is used here to indicate that a material can serve as a source of food to marine bacteria rather than that it suffers rapid visible deterioration. In natural exposure this "attacked" material might perform quite satisfactorily for many years; yet, there is reason to anticipate a longer service life from a more resistant test material. Thus, the accelerated test data are valuable in screening various materials as to their comparative expected performances. When these data are correlated with those from natural marine exposure tests they also establish a gauge for judging new materials. For instance, if BOD results on a new material were similar to those obtained in the laboratory and under natural exposure on rigid unplasticized polyvinyl chloride (PVC), the material would be considered a more available source of carbon than polyethylene but not as utilizable as the elastomers. This becomes the only type of information available until long range tests have been in operation for a considerable length of time.

Polyvinyl chlorides, polyethylene, elastomers, jute, casting compounds, and various synthetic materials have been tested to date, both aerobically and anaerobically. The available oxygen consumed during aerobic tests on elastomeric materials is shown in Fig. 6 (20 C) and Fig. 7 (5 C). Data for PVC plastics and jute are shown in Fig. 8 (20 C) and Fig. 9 (5 C). Total oxygen consumption alone does not permit a comparison of the rates of attack among those materials for which all of the oxygen was consumed. Therefore, the figures also include for each test material, the average oxygen consumption rate, in % per day, determined over the total test period. This is included to provide a measure of the relative rates of attack and is not to imply that the consumption rate was linear with time.

Results from the anaerobic tests are shown in Fig. 10 as ppm of hydrogen sulfide generated over the total test period (16 weeks).

A list of the materials which were tested but which showed no attack by either type of bacteria may be found in Table 1.

To date, the elastomers have been utilized by aerobic bacteria more readily than any other general type of material. The total available oxygen was consumed in all of the ten BOD tests run on elastomers at 20 C (Fig. 6). The time required for complete consumption ranged from 5 to 35 days, giving an average rate of consumption of 19.9 to 2.8% per day. At 5 C all showed attack, but three samples,

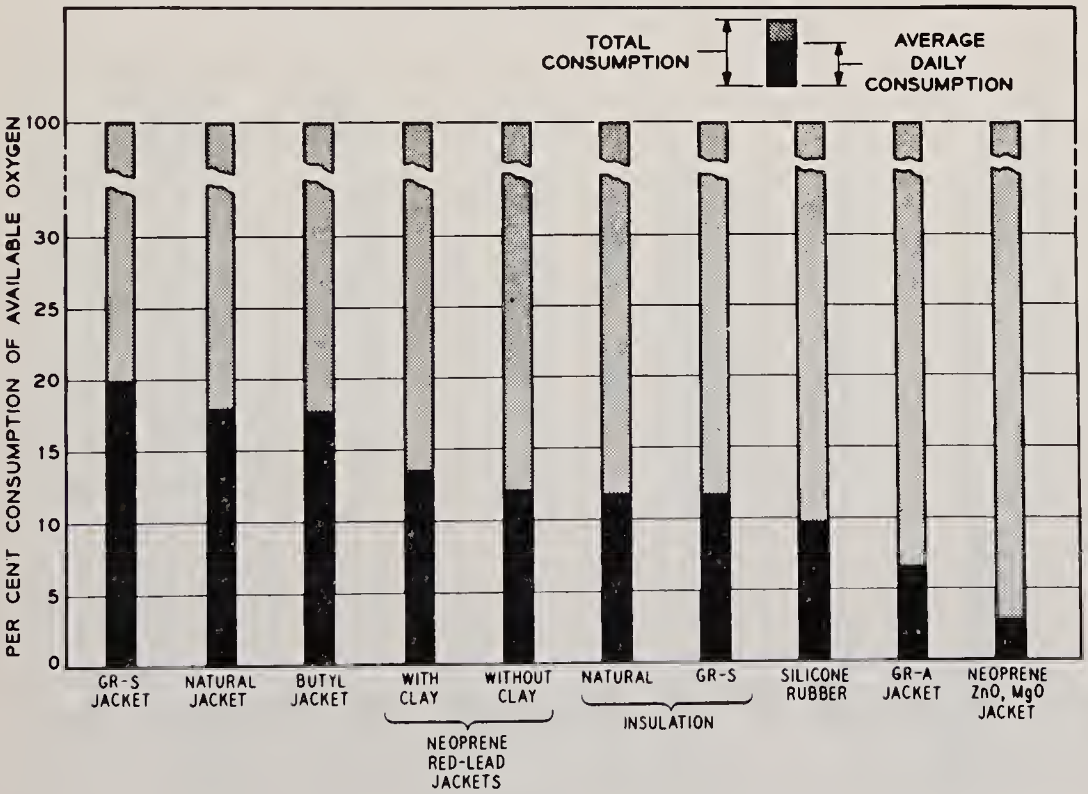


Fig. 6. BOD test on elastomers, 8 weeks at 20 C.

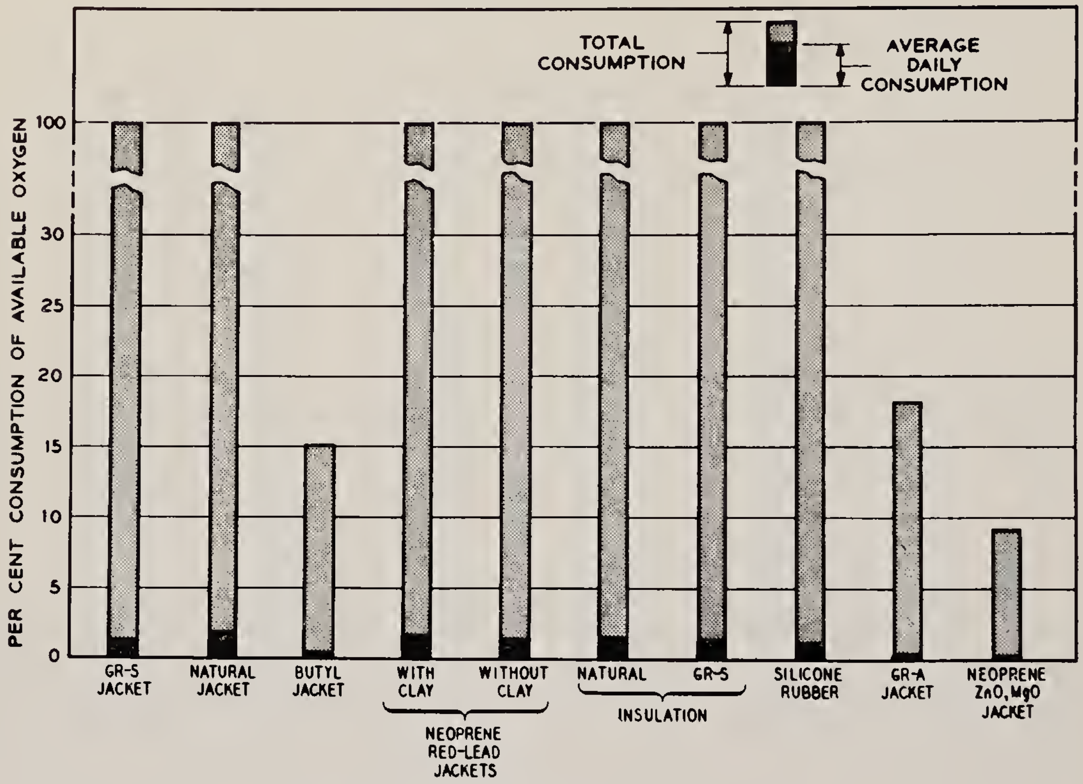


Fig. 7. BOD test on elastomers, 8 weeks at 5 C.

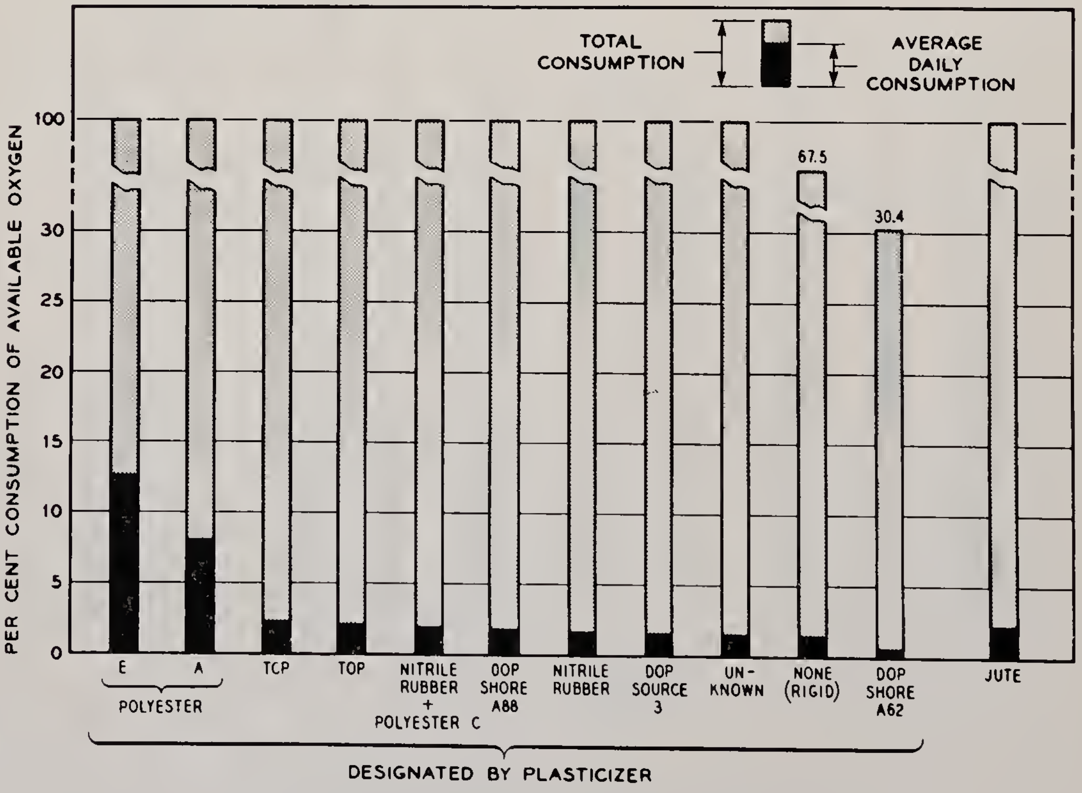


Fig. 8. BOD test on PVC plastics and jute, 8 weeks at 20 C.

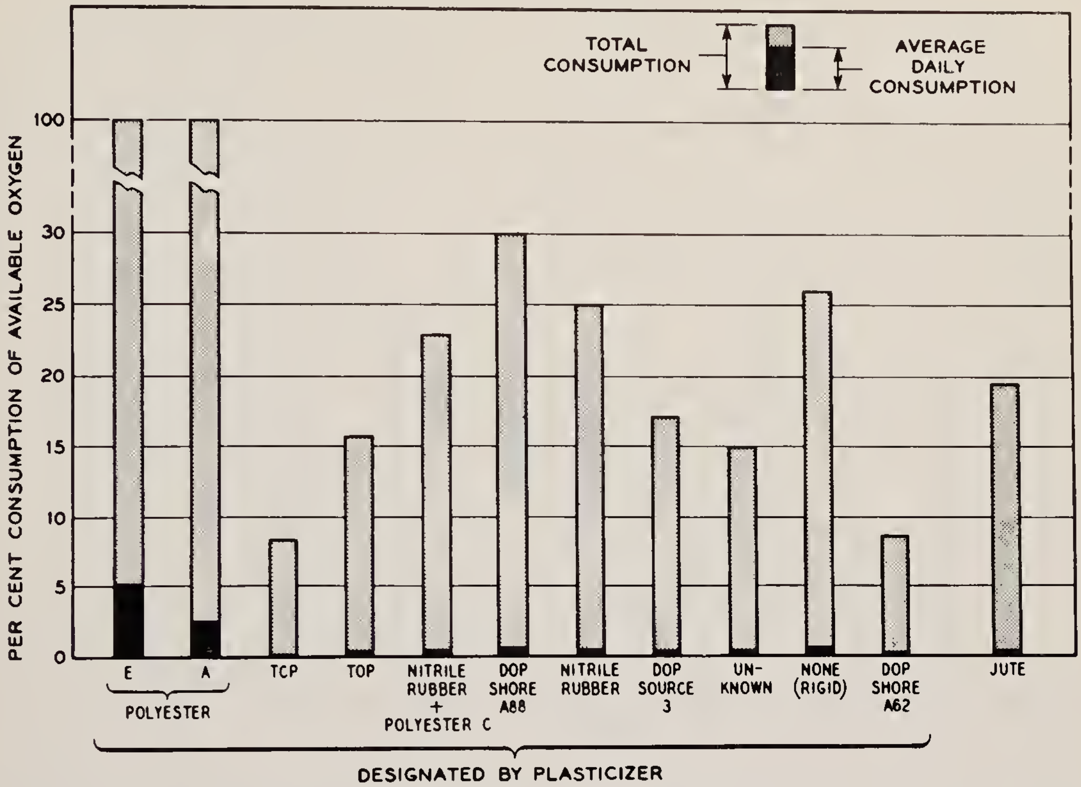


Fig. 9. BOD test on PVC plastics and jute, 8 weeks at 5 C.

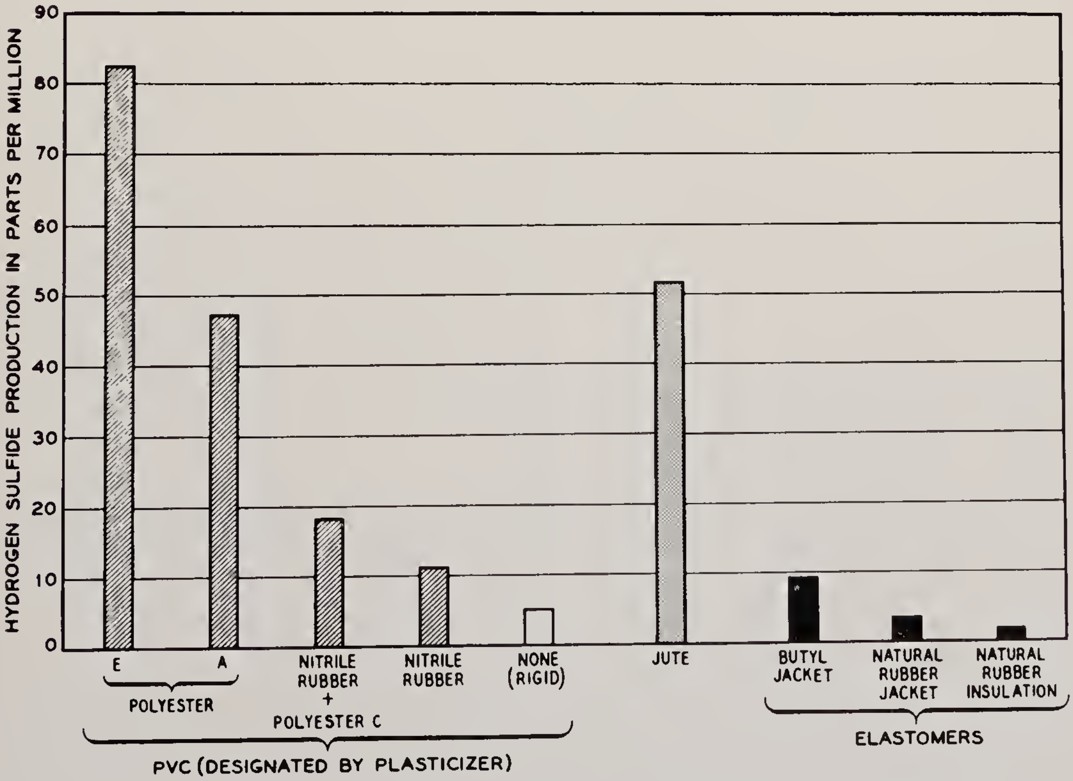


Fig. 10. Anaerobic test results, 16 weeks at 20 C.

Table 1. Materials Not Attacked in Either BOD or Anaerobic Tests

Polyethylene	Polyvinyl chloride
2.0 melt index, standard molecular weight	Semi-flexible copolymer A (no plasticizer added)
0.2 melt index (Source A), high molecular weight	Semi-flexible copolymer B (no plasticizer added)
0.2 melt index (Source B), high molecular weight	Pure PVC resin
0.2 melt index + antioxidant	Other Materials
0.2 melt index + 5% butyl rubber + antioxidant	Nylon (Source A, B, and C)
0.7 melt index (high density) + antioxidant	Mylar
0.7 melt index (high density + carbon black + + antioxidant	Kel F
Polypropylene	Teflon
Polypropylene + antioxidant	Penton
Casting compound	Triacetate cellulose
Epoxide casting compound	Cellulose acetate
	Polycarbonate

namely butyl jacket, GR-A jacket, and neoprene jacket containing zinc oxide and magnesium oxide still showed some resistance following 8 weeks of incubation (Fig. 7). There is some doubt concerning the 5 C results on butyl jacket since it was so readily utilized at 20 C and therefore, this test is being conducted again to check the consistency of these results. Prior to testing the butyl samples an effort was made to remove the protective layer of wax on the samples and perhaps this was accomplished to a lesser degree on one set of samples.

Since the elastomers were compounded with various lubricants, oils, and waxes, and in some cases external plasticizers, it is rather difficult to determine the exact cause for the high rate of microbial activity in these tests. However, it is interesting to note that the rate of oxygen consumption per day varies considerably within the group and also only three of the elastomers, namely butyl and natural rubber jackets and natural rubber insulation, served as a source of energy for the anaerobic bacteria.

The remaining general class of materials which provided a food source for the marine bacteria was the plasticized PVC samples. The data for 20 C are shown in Fig. 8. In all PVC tests that showed high oxygen consumption the materials in question had been compounded with fatty acid type lubricants or external plasticizers that contain fatty acids. Three of the PVC group which were studied and showed no appreciable attack were a pure PVC resin and two internally plasticized semi-flexible copolymers. Another test material which also contained no external plasticizer was susceptible to attack, but this material was compounded with about 8 to 10 times as much fatty acid lubricant as was used in compounding the semiflexible copolymers. These observations indicate that the plasticizer or lubricant and not the PVC itself is utilized as a source of carbon.

As predicted, the rate of attack was much lower in the test incubated at 5 C (Fig. 9). However, the PVC samples plasticized with Polyester E and Polyester A, fatty acid compounds, are attacked considerably faster at both temperatures than the remaining PVC samples. Also, these samples are readily attacked by anaerobes during 16 weeks of incubation (Fig. 10).

One other polyester plasticizer (designated Polyester C) was combined with nitrile rubber for one series of tests and nitrile rubber plasticizer was used separately in another PVC sample. Results of 20 C tests employing both aerobic and anaerobic bacteria indicate that the polyester once again was functioning as the energy source for the bacteria (Fig. 8).

In all tests conducted, jute fiber was readily utilized but it is rather difficult to compare rates of attack on jute with the various sheet materials due to the great variation in surface area available to the bacteria.

Two casting compounds were included in the program, namely, one epoxide and one silica-filled styrene-polyester. There was no microbial activity evident in any of the tests run on the epoxide but the resistance of the styrene-polyester is questionable in the 20 C BOD test, having shown an oxygen consumption of 18.9% at an average rate of 0.3% per day.

The polyethylene samples and other thermoplastic materials such as Mylar, Penton and Teflon were immune to attack in all tests, as shown in Table 1.

Now that the main portion of the BOD and anaerobic test program has been completed attention is being turned to two other proposed areas of study with regard to laboratory testing of the resistance of organic materials to marine bacteria. One of these areas involves the incubation of the materials at low temperature and under pressures approaching those which exist at great ocean depths. The tests are to be run in aged sea water inoculated with barophilic bacteria and ultimately all of the materials will be tested again in an effort to determine which materials would function as a food source for these bacteria.

The second area of study which has been proposed is that of mechanical and electrical testing of the materials after they have been incubated as in the BOD and anaerobic tests. Such data when correlated with the rates of oxygen consumption and hydrogen sulfide production would add considerable valuable information in a rather short period.

In addition to this, BOD and anaerobic tests will continue to be run on new materials submitted for examination. As indicated previously, the data which have been collected to date may find their greatest usage in the future as a basis for anticipating the performance of newly developed materials. Much valuable information can be obtained both by comparing structural similarities in their composition and also their actual performances in BOD and anaerobic tests.

THE OXIDATIVE METABOLISM OF RHODOTORULA AND CRYPTOCOCCUS SPECIES ISOLATED FROM A MARINE ENVIRONMENT

John H. Litchfield and Richard M. Roppel

Biosciences Research, Battelle Memorial Institute, Columbus, Ohio

The physiology of marine yeasts has great interest from the point of view of ecology. These organisms have not been studied sufficiently to permit conclusions about their role in the ocean environment. Recently, Ahearn *et al.* (1960) investigated the use of shaken cultures in the carbon and nitrogen assimilation tests for the identification of a group of marine yeasts isolated from Biscayne Bay, Florida. Their results show a diversity of carbohydrate assimilation patterns among these organisms, especially among species of the genera *Rhodotorula* and *Cryptococcus*. Consequently, it appeared that a study of the oxidative metabolism of selected species of this group of organisms would shed light upon their ecological position in the ocean.

In the study reported in this paper, the growth of *Rhodotorula mucilaginosa* MY-130, *R. graminis* MY-105, *R. texensis* MY-44, *R. minuta* MY-177, and *Cryptococcus laurentii* MY-76 was investigated in both minimal and complex media using glucose, xylose, and gluconate as carbon sources. *R. mucilaginosa, R. texensis,* and *C. laurentii* were selected for further metabolic studies because of the diversity of their assimilation patterns. The oxidation of a variety of carbohydrates and compounds related to oxidative pathways of carbohydrate metabolism by nonproliferating cell suspensions of these organisms was then investigated, and the results of these studies are reported herein.

EXPERIMENTAL METHODS

Cultures of *R. mucilaginosa* MY-130, *R. texensis* MY-44, *R. minuta* MY-177, *R. graminis* MY-105, and *C. laurentii* MY-76 were kindly furnished by Mr. D. G. Ahearn of the Marine Laboratory, University of Miami, Florida. Stock cultures were maintained on the following medium: glucose (anhydrous), 20 g; yeast extract (Difco), 3 g; tryptone (Difco), 5 g; and agar, 20 g per L of medium.

Growth studies and the production of cells for metabolic studies were carried out in shake flasks containing the following ingredients: glucose, xylose, or neutralized glucono-delta-lactone, 20 g; $(NH_4)_2HPO_4$, 5.3 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; NaCl, 0.5 g; $CaCl_2$, 0.23 g; $FeCl_3 \cdot 6H_2O$, 0.005 g; and yeast extract (Difco) 0.3 g per L. The pH was adjusted to 6.5. The carbon sources were sterilized separately to prevent excessive browning of the medium which occurred when all of the ingredients were autoclaved together. The flasks were inoculated with the growth from a 3-day-old agar slant and were incubated on a rotary shaker at 180 2-in. oscillations per min at 28 C.

The minimal synthetic medium used in the growth studies was identical in composition to the shake flask medium with the exception that the yeast extract was omitted. Cultures that were used in these studies were grown on this same synthetic medium to which 2% agar was added.

The cells used for metabolic experiments were harvested by centrifugation after 18 hr of incubation on the rotary shaker, and washed three times with distilled water. They were then suspended in M/45 phosphate buffer, pH 6.5, and agitated in shake flasks for 2 hr at 28 C prior to use in manometric experiments. This treatment lowered the endogenous respiration of the cells without affecting their over-all physiological activity.

The Warburg respirometer was used for measurements of oxygen consumption by resting cell suspensions according to the procedures outlined by Umbreit, Burris, and Stauffer (1957). In all experiments, the gas phase was air and the temperature of the respirometer water bath was maintained at 30 C. The results of preliminary experiments demonstrated that reproducible values for substrate oxidation could be obtained when the endogenous oxygen consumption values were subtracted from those obtained in the presence of substrate.

The concentration of yeast cells in a suspension was determined by centrifuging a 10 ml aliquot, washing the cells with distilled water, and drying the cells at 105 C to a constant weight. Cell concentrations were then calculated as mg dry matter per ml original suspension in manometric studies and as g dry matter per 100 ml original suspension in growth studies.

RESULTS

Growth Studies

The results of previous investigations have shown that *Rhodotorula gracilis* can be grown on a glucose, ammonium salt — mineral salts medium without addition of amino acids or vitamins (Spotholz, Litchfield, and Ordal, 1956). In addition, Nielsen and Nilsson (1950) demonstrated that this organism utilized xylose as a carbon source. Lodder and Kreger-Van Rij (1952) list *R. flava* as the only member of this genus which assimilates lactose. The latter authors do not indicate in their discussion of *Cryptococcus laurentii* whether or not this organism is able to grow on a minimal synthetic medium or is able to utilize pentoses as carbon sources.

Ahearn *et al.* (1960) used Wickerham's Yeast Nitrogen Base medium which contains all of the amino acids and vitamins known to be required by yeasts. However, they did not report any studies of carbon assimilation from a minimal synthetic medium. Thus, it appeared desirable to determine if any of these organisms could assimilate glucose, or xylose from such a medium. All of these yeasts are oxidative and do not ferment sugars; consequently gluconate, which is an intermediate in several oxidative metabolic pathways, was also investigated as a carbon source for these organisms.

The results of this investigation are presented in Table 1. Only *R. graminis* grew on either solid or liquid minimal synthetic media. Glucose, xylose, and gluconate were utilized as carbon sources by this organism in both the minimal and the yeast extract media.

Table 1. Assimilation of Carbon Sources by Marine Yeasts in Minimal and Yeast Extract Enriched Media*

Yeast	Net Grams (Dry Weight)					
	Glucose		Xylose		Gluconate	
	Minimal	Yeast Extract	Minimal	Yeast Extract	Minimal	Yeast Extract
C. laurentii MY-76	-	0.93	-	0.77	-	0.35
R. mucilaginosa MY-130	-	0.90	-	0.45	-	0.15
R. graminis MY-105	0.86	0.96	0.66	0.75	0.31	0.32
R. texensis MY-44	-	0.92	-	0.64	-	0.21
R. minuta MY-177	-	0.86	-	0.24	-	0.12

*The glucose—yeast extract medium was incubated for 4 days. All other media were incubated for 7 days.

C. laurentii assimulated glucose, xylose, and gluconate from the yeast extract medium approximately to the same extent as did *R. graminis*, while *R. texensis* and *R. mucilaginosa* assimilated these compounds to a lesser extent. *R. minuta* did not grow well in the yeast extract medium containing either xylose or gluconate as carbon sources even after a 7-day period of incubation.

In general, these results indicate that marine yeasts vary widely in their nutritional requirements. The B vitamins supplied by the yeast extract in the medium used in these studies might originate in the ocean from the degradation of certain marine algae which contain these growth factors, or from dead marine animal life (Tressler and Lemon, 1951). It would be worthwhile to conduct additional studies of the nutritional requirements of these organisms, especially from the standpoint of requirements for the B group of vitamins.

Glucose, galactose, and pentoses are found as components of the polysaccharides of marine plants, especially in the red and brown algae (Tressler and Lemon, 1951). These sugars might be derived from dead plant tissue by autolytic enzyme action or by the action of enzymes from higher fungi. A study of such nutritional relationships merits consideration and might lead to the isolation of enzymes having economic values.

SUBSTRATE OXIDATION STUDIES

Carbohydrates

On the basis of the results of the nutritional studies and the carbon assimilation data of Ahearn *et al.* (1960), experiments were performed to determine the ability of glucose-grown cells of *C. laurentii, R. texensis,* and *R. mucilaginosa* to oxidize a variety of monosaccharides and disaccharides. Typical results obtained with resting cell suspensions of *C. laurentii* are shown in Figs. 1 and 2; a comparison of net oxygen uptake values for all three organisms is presented in Table 2.

Glucose was oxidized by resting cell of *C. laurentii* at a rapid initial rate that was followed by a second-stage oxidation at a much slower rate. Galactose

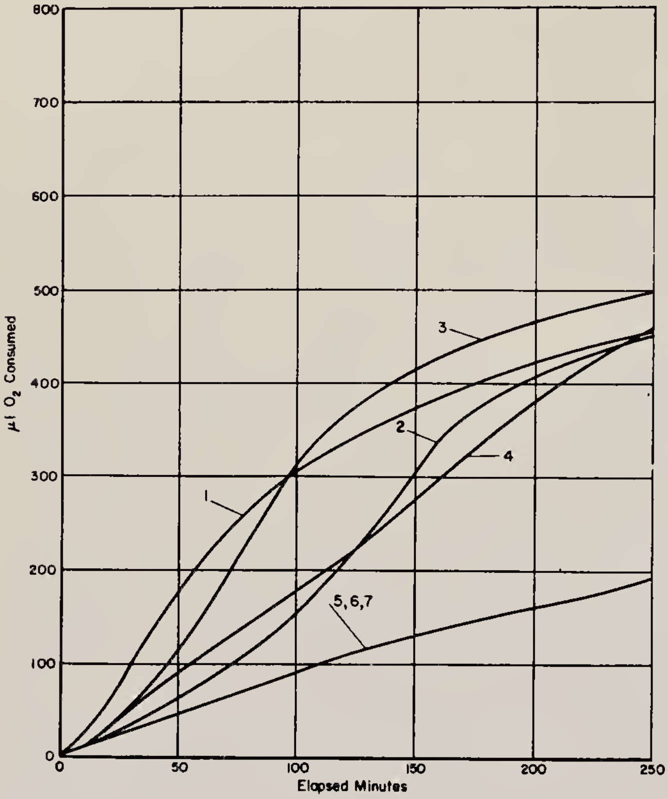


Fig. 1. The oxidation of monosaccharides by resting cell suspensions of *Cryptococcus laurentii.* The Warburg flasks contained 10 μmoles of substrate; 1.0 ml of 0.067 M phosphate buffer, pH 6.5; 1.0 ml of cell suspension (10 mg dry weight); 0.2 ml of 20% KOH in the center well; and water to a total volume of 3.2 ml. 1) Glucose, 2) Galactose, 3) Xylose, 4) D-Arabinose, 5), 6), and 7): Ribose, L-Arabinose, and Endogenous.

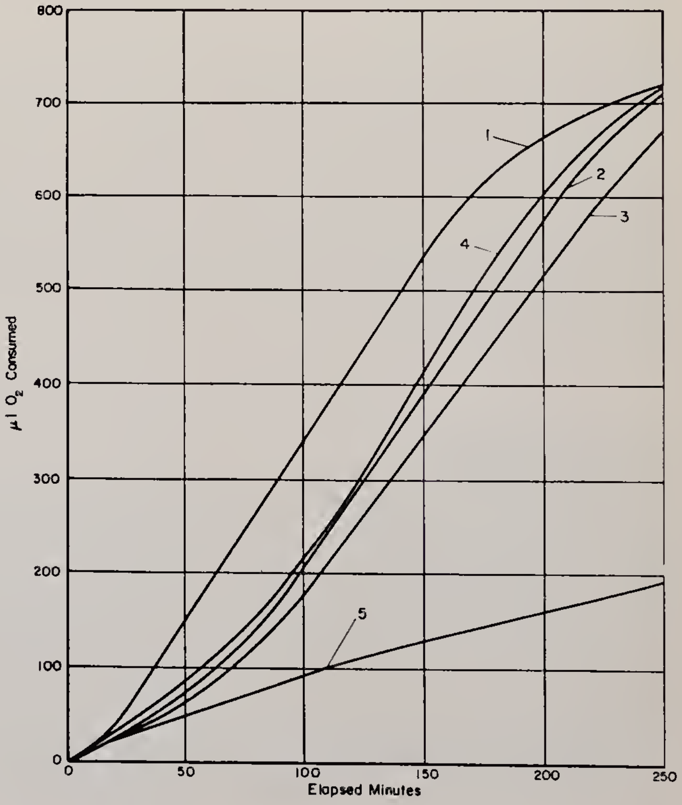


Fig. 2. The oxidation of disaccharides by resting cell suspensions of *Cryptococcus laurentii.* The Warburg flasks contained 10 μmoles of substrate; 1.0 ml of 0.067 M phosphate buffer, pH 6.5; 1.0 ml of cell suspension (10 mg dry weight); 0.2 ml of 20% KOH in the center well; and water to a final volume of 3.2 ml. 1) Sucrose, 2) Maltose, 3) Lactose, 4) Cellobiose, 5) Endogenous.

Table 2. The Oxidation of Various Carbohydrates by Marine Yeasts*

Carbohydrate	*C. laurentii*		*R. texensis*		*R. mucilaginosa*	
	$\mu l\ O_2$	Moles O_2 per Mole Substrate	$\mu l\ O_2$	Moles O_2 per Mole Substrate	$\mu l\ O_2$	Moles O_2 per Mole Substrate
Glucose	238	1.06	218	0.97	212	0.95
Galactose	248	1.11	258	1.15	193	0.86
Xylose	296	1.32	340	1.51	347	1.55
Ribose	21	0.09	294	1.31	10	0.04
D-Arabinose	280	1.25	0	0.00	0	0.00
L-Arabinose	134	0.60	310	1.38	0	0.00
Sucrose	496	2.22	445	1.97	472	2.11
Maltose	492	2.20	0	0.00	0	0.00
Lactose	456	2.04	0	0.00	0	0.00
Cellobiose	476	2.12	0	0.00	0	0.00

*The Warburg flasks contained 10 μ mole substrate; 1.0 ml of 0.067 M phosphate buffer, pH 6.5; 1 ml cell suspension (10 mg dry weight); 0.2 ml 20% KOH in the center well; and water to a total volume of 3.2 ml. Endogenous values were subtracted, and the net values were corrected to 760 mm and 0 C.

was oxidized at approximately the same rate as glucose after a long initial lag period; xylose was oxidized at a more rapid rate than either glucose or galactose, once adaptation had occurred.

D-arabinose was also oxidized to an appreciable extent, but L-arabinose oxidation did not take place until after a lag phase of approximately 4 hr. Ribose was not oxidized in the period of time covered by these experiments.

Resting cells of *R. texensis* oxidized glucose, galactose, xylose, ribose, and L-arabinose but not D-arabinose, while similarly prepared suspensions of *R. mucilaginosa* oxidized glucose, galactose, and xylose, but not ribose, D-arabinose or L-arabinose.

As shown in Fig. 2 and Table 2, the disaccharides sucrose, maltose, lactose, and cellobiose were oxidized by *C. laurentii* to an approximately equivalent extent. A lag period, typical of adaptive enzyme formation, was noted with each of these substrates and was especially pronounced in the case of lactose. This latter curve is quite similar to those obtained with other organisms that are able to adaptively synthesize the enzyme β-galactosidase (Clifton, 1957).

In contrast, sucrose was the only disaccharide oxidized by resting cells of *R. texensis* and *R. mucilaginosa.* Apparently, *R. texensis* does not adapt to oxidize lactose unless it is grown with this substrate as the carbon source. The results of a preliminary experiment showed that lactose-grown cells oxidized both galactose and lactose as well as glucose. Undoubtedly, a similar situation exists in the case of maltose oxidation by *R. mucilaginosa.*

Organic Acids

Since glucose-grown cells of all of these organisms oxidized glucose constitutively and xylose adaptively, the oxidation of compounds related to the oxidative pathways of carbohydrate metabolism and the Krebs' tricarboxylic acid cycle was investigated. The results of this study are presented in Table 3.

Table 3. The Oxidation of Various Organic Acids by Marine Yeasts*

Organic Acid	*C. laurentii*		*R. texensis*		*R. mucilaginosa*	
	$\mu l\ O_2$	Moles O_2 per Mole Substrate	$\mu l\ O_2$	Moles O_2 per Mole Substrate	$\mu l\ O_2$	Moles O_2 per Mole Substrate
Gluconate	264	1.18	20	0.09	34	0.15
2-Ketogluconate	128	0.57	0	0.00	0	0.00
Acetate	173	0.78	116	0.52	102	0.46
Pyruvate	230	1.03	126	0.56	158	0.71
α-Ketoglutarate	38	0.17	73	0.33	0	0.00
Succinate	46	0.21	49	0.22	29	0.13
Fumarate	24	0.11	32	0.14	64	0.29
Malate	0	0.00	51	0.23	0	0.00
Citrate	33	0.15	35	0.16	0	0.00

*The Warburg flasks contained 10 μ mole substrate; 1.0 ml of 0.067 M phosphate buffer, pH 6.5; 1 ml cell suspension (10 mg dry weight); 0.2 ml 20% KOH in the center well; and water to a total volume of 3.2 ml. Endogenous values were subtracted, and the net values were corrected to 760 mm and 0 C.

C. laurentii oxidized gluconate and 2-ketogluconate to a substantial extent after a period of adaptation. *R. texensis* and *R. mucilaginosa* oxidized gluconate poorly and did not oxidize 2-ketogluconate. The ability of resting cells of *C. laurentii* to oxidize 2-ketogluconate is of interest since this compound is found in certain red algae, notably *Chondrus crispus* (L) Stack. (Tressler and Lemon, 1951). It is possible that in the natural habitat of *C. laurentii* this compound derived from decaying red algae serves as a carbon source.

Acetate and pyruvate were oxidized by resting cells of all three organisms. The 1.0 mole of oxygen consumed by *C. laurentii* with pyruvate as a substrate is equivalent to that amount required for the oxidation of this substrate to the acetate stage.

Glucose-grown resting cell suspensions of these organisms did not oxidize any of the tricarboxylic acid cycle compounds to a significant extent. However, it cannot be concluded that the tricarboxylic acid cycle is not operative in these organisms since Ajl (1951) has pointed out that inability to oxidize these substrates may result from cellular permeability properties. Studies with cell-free extracts would be necessary to determine the presence of the tricarboxylic acid cycle.

DISCUSSION

None of the carbohydrates or organic acids investigated in this work were oxidized to completion by any of these marine yeasts. Similar results have been observed with other microorganisms and suggest that these substrates were oxidatively assimilated into cellular material (Clifton, 1946).

It is interesting to note the differences in substrate oxidation patterns exhibited by *R. gracilis* NRRL Y-1091 of terrestrial origin, in comparison with the marine *Rhodotorulae* and *C. laurentii.* Litchfield and Ordal (1958) found that glucose-grown cells of *R. gracilis* NRRL Y-1091 oxidized glucose, xylose, D-arabinose, L-arabinose, gluconate, acetate, pyruvate, and all of the dicarboxylic acids of the tricarboxylic acid cycle. Evidence for the oxidative assimilation of all these compounds was also presented. A further comparison of these organisms indicates that the marine yeasts studied herein are oxidatively less active than the terrestrial strain of *R. gracilis.* Dissolved oxygen concentrations in the sediments from which these marine yeasts were isolated may be low enough to favor the success of organisms having slower metabolic reactions.

In general, the marine yeasts investigated show a wide diversity of substrate assimilation and substrate oxidation patterns. The adaptive oxidation of galactose and xylose indicate that these substrates, if liberated from marine plant polysaccharides, could be metabolized by these organisms. The oxidation of cellobiose by glucose-grown cells of *C. laurentii* is interesting in that cellobiose is the product of cellulose degradation by many fungi. Apparently, cellobiase activity in this organism may be induced in an environment where cellulosic materials are being hydrolyzed by marine fungi.

Further investigations are needed to demonstrate the mechanisms operative in the oxidation of various substrates by marine yeasts. An ecological study of the possible association of these organisms with higher marine fungi might provide an over-all picture of their role in the oceans.

SUMMARY

The growth of *Cryptococcus laurentii* MY-76, *Rhodotorula mucilaginosa* MY-130, *R. graminis* MY-105, *R. texensis* MY-44, and *R. minuta* MY-177 was investigated in a minimal synthetic medium and in this same medium enriched with yeast extract, using glucose as the carbon source. Only *R. graminis* was able to grow in the minimal synthetic medium, while all of these organisms grew rapidly in the yeast extract medium.

C. laurentii, R. graminis and *R. texensis* assimilated both xylose and gluconate, while *R. mucilaginosa* assimilated only xylose. *R. minuta* did not assimilate either xylose or gluconate to a significant extent.

Glucose, galactose, and xylose were oxidized by glucose-grown cells of *C. laurentii, R. mucilaginosa* and *R. texensis. C. laurentii* oxidized both D- and L-arabinose, but did not oxidize ribose, while *R. texensis* oxidized ribose and L- but not D-arabinose. *R. mucilaginosa* did not oxidize any of these pentoses. Sucrose, maltose, lactose, and cellobiose were oxidized by resting cells of *C. laurentii* but only sucrose was oxidized by similar cell preparations of *R. texensis* and *R. mucilaginosa.*

Gluconate and 2-ketogluconate were oxidized by glucose-grown cells of *C. laurentii*, but *R. texensis* and *R. mucilaginosa* did not oxidize either of these compounds to any significant extent. Resting cells of all of these organisms oxidized acetate and pyruvate but none of these preparations oxidized any of the acids of the tricarboxylic acid cycle to any appreciable extent. These results were discussed with reference to the physiological characteristics reported in the literature for *R. gracilis*, a terrestrial organism, and with reference to the presence of these various substrates in marine plants.

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PRESIDENTIAL ADDRESS

THE ROAD WE MUST TRAVEL

Charles C. Yeager, President
Society for Industrial Microbiology, 1959-1960

Scientific Oil Compounding Co., Inc., Chicago, Illinois

An old man and his son were travelling on a mountain trail with only one horse between them. Since walking was difficult for the father, it was decided that it would be better for him to ride the horse and the young man to walk. After a while they met a traveller. When he saw the old man riding, he cried, "Why do you make the young man walk? Don't you know that his legs will soon be stiff like yours and even may become crippled? Why don't you get off the horse and let him ride?" The old man thought that this made sense, so he got off and let his son ride. A little further on they met another traveller, who thought it awful that the young man should ride and let his poor old father walk, and he told them so. The son agreed, and both father and son mounted the horse. Then they ran into a third traveller. "I ought to report you to the SPCA," he said, "two big men riding one poor horse." This made the man and his son ashamed of themselves, so they got down from the horse and walked, leading him. Soon they met a fourth traveller. When he saw two men leading an idle horse he exclaimed, "Are you crazy? Why walk along a difficult trail when you could just as easily ride?"

The new president of the Society will soon appreciate this little story, because he'll have some people disagreeing with him, no matter what he may do. I had my share of this trouble, and I gladly unload it on his shoulders for the coming year.

Nevertheless, as my term of office approaches its close, I confess that my tenure has had its happy moments too. I would first like to express my deep gratitude to those who made my path easier, in particular, Drs. Kaplan, Miller, and Dulaney of our Board of Directors. They helped a lot with their enthusiastic and unqualified support and most able assistance. Without them, my job of president would have been a nightmare.

Nor can I forget the excellent work of our committees and the unstinting labors of each chairman. I would like personally to thank all of them: Miles Sharpley of our Program Committee; Ray Allred and Bob Huddleston, Local Arrangements Committee; Dave Baskin, Education Committee; Mary O'Hara, Publicity Committee; Will Hoefen, Nominations Committee; Ernest Walker, Constitution Revision and Society Incorporation Committee; Walter Bejuki, AAAS representative; and Lloyd Herman, Chairman of our newest Committee on Antimicrobial Agents.

There are, of course, many others to whom we all owe a debt of gratitude, because they too labored for the cause and furthered the aims of our Society.

So much for bouquets; now for some rocks. I herewith hand an official reprimand to those members to whom tasks were assigned, but who did nothing. Quite a number of them requested committee jobs, but although only a limited amount of their time was required, they reneged. Shall I cite them by name, or may I recall to you the story of the English lord, who found himself in a similar predicament ? The Queen, in the presence of the whole court, ladies included, asked him to name the three women he considered the most beautiful in England. "Your Majesty is the first," he replied. "The second is my wife. The third one knows very well that I mean her, so I will not publicly declare her identity."

Seriously speaking, the president's job becomes exceedingly difficult, if he finds that he can't rely on his committee members, and must either do the work himself or appoint others at late dates. A society is only as strong as the activities of its members. If their activities are lethargic, the society remains lethargic. It's a poor society that has to depend entirely upon the activities of but a few of its members. As outgoing president I urge the entire membership to take an active part in Society affairs. We need new workers—new blood to take over the administration of our future programs. *This means all of you.* It is only in this way that we can grow and exert a greater influence in the microbiological field.

One of our newest activities this past year concerned an Education Survey, in which colleges and universities from coast to coast have participated. For several years other scientific societies have urged a modernization of our curricula, but unfortunately in all instances these efforts have been limited to the high school level. Collegiate curricula have been generally ignored, since few have cared to criticize institutions of advanced learning for failing to meet the challenge of the nuclear and space age. Since the Society for Industrial Microbiology has as one of its aims the fostering of cooperative efforts between academic institutions and industry, we have deemed it essential to provide academic organizations with pertinent information as to the type of students industry wishes to see developed in our colleges and universities. Accordingly, a committee was appointed first to survey existing curricula, and second to survey industry to determine their individual requirements in the microbiological area.

Questionnaires were sent out to some forty colleges and universities across the country. Cooperation was excellent, and most of the questionnaires were returned. A report was prepared, in which the data so collected were evaluated. It is about some of the interesting aspects of this survey that I would like to say a few words.

Industry for a long time has found it necessary to establish elaborate training programs for men recently recruited directly from academic schools. Must this be so ? These industrial training programs are time-consuming and extremely expensive, but could be obviated if the student were oriented to practical industrial problems during his collegiate preparation. After all, this is not a new idea. Colleges have been graduating students for years in various lines of engineering, and most of these men were admirably trained for work in their chosen fields. Why must the microbiologist remain such a "blue-nose" ? To be brutally frank, the B.S. degree no longer means much more than the paper on which it's printed so far as its value to industry is concerned, and yet it is from this vast group of B.S. graduates that it must draw most of its professional workers. The statistics developed through the tabulation of the survey results showed that our Bachelor of Science graduates are totally unprepared for anything other than jobs as technicians, that they must be trained for at least two additional years before they are of much use, and, saddest of all, that our colleges and universities as a whole do not seem interested in training their microbiology undergraduates for industrial positions. Perhaps this is due to a desire on the part of the academic staffs to impart a general, well-rounded, basic education to the student—certainly an admirable and understandable objective. But, in addition, and far too often, the faculty members themselves are not aware of new developments in industrial progress.

Of course, there are exceptions to the rule. It is but natural that technological colleges are leading the procession of this minority. To a much lesser extent some of our State universities are beginning to take interest in industrial problems. And in one admirable high institution of learning we were even asked to inform them of industry's needs, so it could take steps to modify the curricula accordingly.

Unfortunately, however, in other colleges the staff showed no interest at all in industrial problems, made no attempt to keep up with latest industrial technology, and did not encourage their students to seek industrial positions, in fact freely admitted that it had no idea what happened to their students after graduation.

In the survival age in which we live, this is not only a professional, but a national problem of vital importance, for we must be ready to meet Communistic teams both on the war and the industrial front, and we cannot beat them with insufficiently trained personnel.

What can our Society do to provide the needed remedy? If you will recall, there were to be two phases of this survey program—a survey of colleges and a survey of industrial needs. We now have a good idea as to the collegiate situation. While many of the colleges believe that standards in general should be broadened, only 10% claim that industrial orientation is not appropriate for undergraduates. We must actively press for curricula changes. Redundant course work should be eliminated and the released time given over to technological courses in the student's major.

These changes are not going to be easily accomplished. It's going to take months, even years of intensive survey and work to make a dent in our present educational processes. But we have no time to spare; in fact, we have been dawdling too long already. To facilitate this change, we ought to, among other things, consider the establishment of a Speaker's Bureau, probably implemented through our local sections. It is imperative that the speakers be drawn from industrial and governmental groups specializing in the various phases of microbiology. The speakers must be lucid, interesting, and enthusiastic so as to impart a desire on the part of the student to consider the practical as well as the basic phases of microbiology in preparing for graduation. Educational institutions today are demanding more and more of the support of industry. It is only right therefore that industry have something to say in the way its future employees are to be trained. There is no doubt that students and faculty members alike would learn much from speakers drawn from industry. For years Purdue University has conducted a series of symposia in which, not only basic philosophies are discussed and studied, but at the same time practical aspects are presented by industrial experts. This is a step in the right direction; nevertheless, we believe that this idea should be expanded so as to make this marriage of academic and practical training a four-year curriculum.

Perhaps we may find the job too big for one organization alone. It is possible that we may have to invite the participation of other microbiological societies. It is also possible that subsequently the entire programs be implemented by the American Institute of Biological Sciences. Under any circumstances, if we are to live up to the lofty aims of our Society, the job must be done.

There will be many obstacles in our path, because academic organizations do not as a rule accept gracefully the recommendations of outsiders. Many colleges will continue to insist on subordinating the B.S. degree to a full graduate program. We certainly should encourage the expansion of active graduate programs; admittedly, more Ph.D.'s must be developed; but at the same time we must urge the modernization of our training programs so as to better prepare the graduates who will go no further than the B.S. degree. This is the foundation upon which our future industrial progress is being built. This is the group that will determine whether in the future our technological system will win or lose the struggle against Communism, in which, on the industrial front, we are already heavily engaged.

The new administration will take over almost immediately. I urge that this worthwhile program be continued. The second phase, an active survey of industrial needs, should be undertaken, and the data passed on to the colleges. This will be a monumental task, but it must be done. In the meantime, broadening of curricula in the way indicated, even if only on a tentative basis, should not be postponed. The Education Committee must be ready to submit such a program on demand.

I have great confidence in the membership of the Society for Industrial Microbiology. We have become recognized as most progressive in our thinking. We have the capacity for exerting profound influence in the various fields of microbiological sciences. We must press forward with continuing vigor so as to bring credit to ourselves, our industry, and our Society.

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